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Functional and biological characterization of SSC5D, a novel molecule of the Scavenger Receptor Cysteine-rich family

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

CATARINA BESSA PEREIRA

FUNCTIONAL AND BIOLOGICAL CHARACTERIZATION OF SSC5D, A NOVEL MOLECULE OF THE SCAVENGER RECEPTOR CYSTEINE-RICH FAMILY

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor Alexandre Carmo

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Afiliação – Instituto de Biologia Molecular e Celular, Instituto de Investigação e Inovação em Saúde, e Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Legal precepts

De acordo com o disposto no nº 2, alínea a), do artigo 31º do Decreto-Lei no 230/2009, utilizaram-se neste trabalho resultados já publicados, que a seguir se discriminam:

Bessa Pereira, C., Bocková, M., Santos, R.F., Santos, A.M., Martins de Araújo, M., Oliveira, L., Homola, J., and Carmo, A.M. (2016). The Scavenger Receptor SSc5D Physically Interacts with Bacteria through the SRCR-Containing N-Terminal Domain. Frontiers in Immunology 7 (article 416).

A autora desta dissertação, Catarina Bessa Pereira, declara que participou em todo trabalho experimental desta tese de doutoramento, com a excepção das experiências que envolveram técnicas de imunohistoquímica (realizadas por Rita Santos) e na experiência representada pela figura 2 do chapter 2 (realizada por Liliana Oliveira).

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...e a ti, Pedro "com um brilhozinho nos olhos".

Abstract/Resumo

Abstract

To overcome potential harmful threats from microbes, our immune system relies on complex mechanisms that involve interactions between diverse cells and proteins. Nowadays, growing evidence suggests that the ancient and highly conserved group of proteins belonging to the Scavenger Receptor Cysteine-Rich (SRCR) superfamily display a great functional variability, indispensable for the proper function of the immune system. This heterogeneous group is composed of membrane and soluble extracellular proteins that are expressed in various cell types, and some were found to mediate bacteria binding and possibly to contribute to the eradication of virulent agents. Human SSC5D is a soluble glycoprotein of the SRCR superfamily that consists of two structurally distinct parts: an N-terminal domain, which contains five SRCR domains, and a C-terminal mucin-like domain. In some members of this superfamily, the SRCR domains were shown to mediate protein-protein interactions and pathogenic recognition, suggesting that SSC5D can have a role as an innate sensor for pathogens. To better understand the function of SSC5D, we produced the two domains independently to screen for putative interactions with cell surface receptors and to explore the ability of SSC5D to bind bacteria through its SRCR domains. To further characterize the ability of N-SSC5D to interact with bacteria, we developed a novel and more sensitive approach using a surface plasmon resonance (SPR)-based method. Our results showed that despite no interactions with cell surfaces were observed at this stage, N-SSC5D could interact with bacteria, which makes this protein a potential receptor for bacterial sensing. Moreover, our studies of native SSC5D tissue distribution using a new antibody showed that SSC5D is highly expressed by epithelial cells at the interface of mucosal tissues and in placenta. The SSC5D privileged localization suggests an active role in the protection of mucosa from pathogens and at the maternal-foetal interface. These body locations are extremely vulnerable to infection and inflammation; therefore, to perceive the biological relevance of SSC5D we initiated a process of generation of a mouse deficient for the SSC5D gene.

Resumo

Para superar possíveis ameaças de agentes patogénicos, o nosso sistema imunitário depende de um conjunto de mecanismos complexos que envolvem interações entre diversas células e proteínas. Estudos recentes sugerem que os membros da antiga e altamente conservada família "Scavenger Receptor Cysteine Rich (SRCR)", apresentam uma grande diversidade funcional, indispensável para um correto funcionamento do sistema imunitário. Estas proteínas, que podem ser membranares ou solúveis, são expressas em diversos tipos de células e formam no seu conjunto um grupo bastante heterogéneo, onde alguns membros se ligam a bactérias, possivelmente contribuindo para a sua erradicação. A glicoproteína SSC5D humana é um membro solúvel da superfamília SRCR, que consiste em duas partes estruturalmente distintas: um domínio N-terminal, que contém cinco domínios SRCR e um domínio C-terminal semelhante a uma mucina. Noutros membros desta superfamília, os domínios SRCR, mostraram-se capazes de mediar interações proteína-proteína e de detetar agentes patogénicos, sugerindo que o SSC5D pode também agir como um sensor inato para agentes patogénicos. Assim, para se compreender melhor a função do SSC5D, produzimos de forma independentes os dois domínios com o intuito de encontrar possíveis interações com recetores na superfície celular, e explorar a capacidade do SSC5D se ligar a bactérias através dos seus domínios SRCR. Adicionalmente, para se caracterizar com maior profundidade a capacidade do SSC5D se ligar a bactérias, desenvolvemos uma nova abordagem mais sensível que envolve a aplicação da técnica de ressonância de plasma de superfície (RPS). Os nossos resultados mostraram que, apesar de não termos observado nenhuma interação com a superfície das células nesta fase inicial, o domínio N-terminal da proteína SSC5D consegue interagir com bactérias, o que torna esta proteína é um potencial recetor para deteção de bactérias. Adicionalmente, utilizamos um anticorpo novo para analisar a distribuição da proteína nativa em diversos tecidos humanos. Nestes estudos observamos que o SSC5D é altamente expresso nas células epiteliais que se encontram na interface dos tecidos da mucosa e na placenta. Esta localização privilegiada sugere, que o SSC5D pode ter um papel ativo contra agentes patogénicos nas mucosas e também na interface materno-fetal. Como estes locais do organismo são extremamente vulneráveis à infeção e inflamação, iniciamos um processo para gerar um ratinho sem o gene SSC5D com o objetivo de se compreender melhor a relevância biológica do SSC5D.

Abbreviations

AA	amino acid
Ab	Antibody
Ag	Antigen
AIM	Apoptosis Inhibitory factor expressed by Macrophages
ALCAM	Activated leukocyte cell adhesion molecule
APC	Antigen Presenting Cell
BCR	B cell receptor
bp	base pair
BSA	Bovine Serum Albumin
C-terminal	Carboxy-terminal
CD	Cluster of Differentiation
CLR	C-type lectin receptors
CUB	Complement C1r/C1s, Uegf, Bmp1
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DMBT1	Deleted in Malignant Brain Tumours
EGF	Epidermal growth factor
FACS	Fluorescently-activated Cell Sorting and Analysis
FCS	Foetal Calf Serum
HA	Hemagglutinin
His	Histidine
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IFN	Interferon

IHC	Immunohistochemistry
IL	Interleukin
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine based activation motif
IP	Immunoprecipitation
IS	Immunological Synapse
ITIM	Immunoreceptor tyrosine based Inhibitory Motif
Kd	Dissociation Constant
kDa	Kilodaltons
KO	Knockout
LAT	Linker for activation of T cells
LFA-1	Leukocyte function-associated antigen
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M160	Scavenger receptor cysteine-rich type 1 protein M160
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinases
MHC	Major Histocompatibility Complex
MW	Molecular weight
N-terminal	Amino-terminal
NF-κB	nuclear factor kappa B
NK	Natural killer
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domains
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood lymphocyte(s)

PBMC	Peripheral blood mononuclear cell(s)
PE	Phycoerythrin
PGN	Peptidoglycan
Pro (P)	Proline
PRR	Pattern recognition receptor
RLR	Retinoic acid-inducible gene (RIG)-I-like receptors
Ser (S)	Serine
SID	SRCR Interspersed Domains
SP	Surfactant proteins
SPR	Surface plasmon resonance
SR	Scavenger receptor
SRCR	Scavenger receptor cysteine rich
SSC4D	Scavenger receptor cysteine rich family member with 4 domains
SSC5D	Soluble Scavenger with 5 SRCR Domains
TCR	T cell receptor
TCS	Tissue culture supernatant
Th	T helper cell
Thr (T)	Threonine
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TWEAK	TNF-like inducer of apoptosis
Tyr	Tyrosine
WB	Western blot
WT	Wild-type
ZP	Zona pellucida

General Introduction

1. The immune system

The second we are born and throughout our lifetime we continuously interact with trillions of microbial species (Bulek et al., 2010; Hancock et al., 2012). These include commensal bacteria that colonize the mucosal surfaces of our body and provide us with vital benefits such as products of the metabolism of nutrients in gut (Bulek et al., 2010), but also pathogenic agents that can become lethal if not efficiently neutralized. The evolution of our immune system was therefore driven by the need to balance all these interactions so we can have an effective system that eliminates or neutralizes pathogens while preserving beneficial mutualistic relations (Hooper et al., 2012; Ayres, 2016). Not surprisingly, the dysregulation of such a complex system often results in life-threatening infectious and inflammatory diseases (Takeuchi and Akira, 2010; Hancock et al., 2012; Hooper et al., 2012). At the same time, it is well established that even in the absence of an infectious organism, an altered or inappropriate activity of the immune system contributes to other pathologies such as cancer and autoimmune disorders (Crespo et al., 2013; Lu, 2013; Nishikawa and Sakaguchi, 2014; Singh et al., 2014; Coffelt et al., 2015; Nelson et al., 2015).

The immune system can be broadly divided into innate and adaptive arms that are nevertheless highly interdependent, and often effective responses encompass components of both systems (Flajnik and Du Pasquier, 2004; Hancock et al., 2012). Interestingly, as new immune mechanisms are being revealed such as 'trained immunity' properties in NK cells and macrophages, the distinction between the two arms is becoming less clear (Flajnik and Du Pasquier, 2004; Litman et al., 2010; Netea et al., 2011).

1.1. Microbial recognition by the immune system

The main distinction between innate and adaptive immunity resides in the type of receptors used to recognize pathogens (Fig. 1) (Medzhitov, 2007). Innate cells sense microorganisms directly through pattern recognition receptors **(PRR)** that recognize 'immutable' and defined microbial signature molecules, also known as pathogen-associated molecular patterns **(PAMP)** that are foreign to the host (Janeway Jr and Medzhitov, 2002). These include bacterial and fungal cell wall components or viral nucleic acids (Littman 2010, Iwasaki 2015). PRR are germline-encoded and collectively enable the recognition of a wide universe of microbes (Litman et al., 2010). An even

larger universe of structures is recognized by B and T cells. These express unique types of functional receptors, generated by **random genetic recombination**, that recognize a particular **antigen**. Antigenic challenge leads to the activation and clonal expansion of monotype lymphocytes expressing a specific receptor, which results in the generation of both effector and 'memory' cells (Mahnke et al., 2013; Kugelberg, 2015).

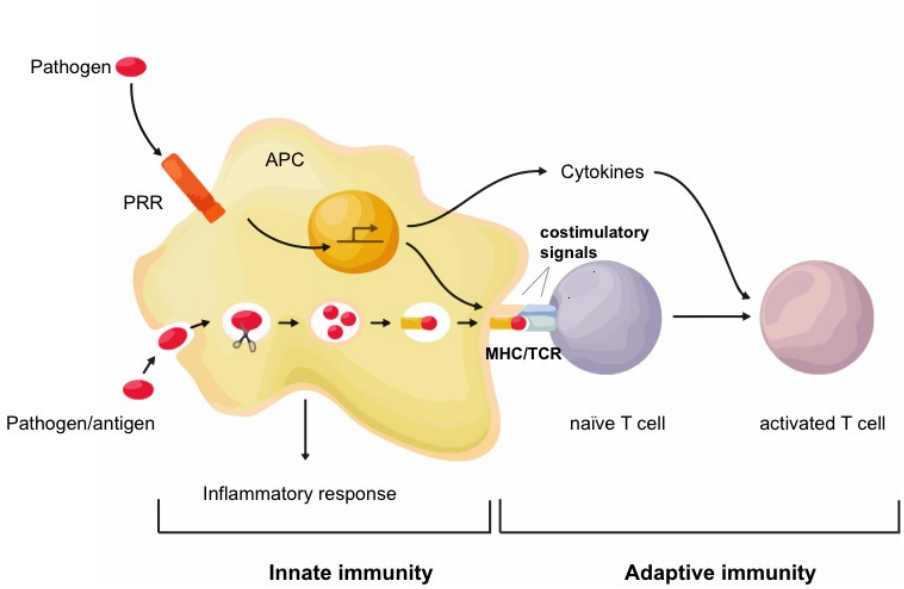

Figure 1. Innate and adaptive immunity (Source: Kobayashi K., 2017; Retrieved from http://research4.dfci.harvard.edu/innate/innate.html). Innate immune cells include epithelial cells, phagocytic cells (macrophages and neutrophils), natural killer (NK) cells and dendritic cells (DC) that rapidly recognize conserved microbial structures through pattern recognition receptors (PRR). The specialized components of the immune system, the T and B lymphocytes, are activated trough their cell surface receptors (T- or B- cell receptor) that recognize specific antigenic structures. APC: Antigen-presenting cell; MHC: Major histocompatibility complex; TCR: T- cell receptor.

1.1.1. Innate immunity and PRR

Microbial nonself recognition by PRR is an evolutionary conserved strategy used by a wide range of organisms to activate diverse cellular defence mechanisms. In response to virulent agents, innate cells produce pro-inflammatory cytokines, which alert other host cells to the occurrence of infection, promote recruitment of immune cells to the site of infection, stimulate cell differentiation and induce microbicidal mechanisms, like the production of reactive oxygen species (Janeway Jr and Medzhitov, 2002; Hancock et al., 2012). In addition, during infection microbe-invaded cells can emit danger or

stress signals, which are sensed by PRR (Rivera 2016). As a consequence, PRR signalling triggers the expression of differential cytokine secretion to promote pathogen eradication (Fig. 2) (Rivera et al., 2016).

Toll-like receptors (TLR) are the first and foremost studied class of PRR. Initially identified on the basis of sequence similarity with the Drosophila protein Toll, TLR are evolutionary conserved type 1 transmembrane proteins found in vertebrates, invertebrates and plants. Members of the TLR family have a common cytoplasmic domain designated Toll-1L-1R, or TIR domain, responsible for signal transduction, and an amino-terminal extracellular leucine-rich repeat (LRR) domain (Armant and Fenton, 2002). Each member recognizes different microbial structures through differences in their extracellular domain (Akira et al., 2001; Armant and Fenton, 2002). TLR may be located on the cell surface or in the endosomal compartment and their expression is modulated in response to various stimuli (Akira et al., 2001).

Evidence that TLR were PRR involved in mammalian innate immune defence stemmed from the discovery that a mouse strain hyporesponsive to lipopolysaccharide (LPS) carried a loss-of-function mutation in the TLR4 gene, thus establishing TLR4 as a putative receptor for LPS of Gram-negative bacteria (Poltorak et al., 1998; Armant and Fenton, 2002). This observation was confirmed by the generation of $TLR4^{-/-}$ knockout (KO) mice that could not trigger LPS-driven responses. However, overexpression of TLR4 *in vitro* was not sufficient to trigger an LPS-induced response, which suggested that other molecules were involved in LPS recognition. One such molecule was found to be the secreted MD2 protein that physically associates with the extracellular domain of TLR4 to recognize LPS in the presence of CD14, already described as binding LPS (Kim et al., 2005). Interestingly, the TLR4-MD2 complex is also responsible for the recognition of viral proteins and endogenous host proteins that result from stressed or damaged cells such as endogenous HSP60 and fragments of the extracellular matrix components like fibronectin (Ohashi et al., 2000; Okamura et al., 2001; Vabulas et al., 2001; Mollen et al., 2006)

Further studies using various KO mice challenged with diverse pathogens clarified the function of other TLR. Transmembrane TLR typically recognize microbial cell wall components or virulence factors. For instance, TLR2 in conjunction with TLR1 or TLR6 recognize lipopeptides, Gram-positive bacteria and fungal cell wall components while TLR5 recognizes flagellin. Conversely, endosomal TLR sense microbial nucleic acids. TLR3 recognizes double-stranded RNA, TLR7 and TLR8 bind single-stranded RNA, and TLR9 recognizes unmethylated CpG dinucleotides (Hancock et al., 2012; Broz and

Monack, 2013). TLR signalling ultimately leads to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling, as well as IFN-regulatory factors (IRFs) (Hatada et al., 2000). These pathways induce pro-inflammatory cytokines, chemokines and type I interferons (IFN) resulting in the activation of cellular antimicrobial functions such as phagocytosis, oxidative burst and production of antimicrobial peptides (Hancock et al., 2012).

Besides TLR, other families of PRR are also known to have relevant roles in pathogen recognition (Akira et al., 2001; Kawai and Akira, 2011). C-type lectin receptors (CLR) are a heterogeneous group of transmembrane (*e.g.*, dectins, mannose receptor) or soluble proteins (*e.g.*, *co*llectins) with C-type lectin domains that recognize a wide range of carbohydrate ligands. These include β-glycans, mannose and fucose from fungi and bacteria, but they can also bind non-carbohydrate ligands (Cambi et al., 2005). Transmembrane receptors are mainly expressed on immature dendritic cells and macrophages and can induce signalling pathways that directly activate NF-κB, to promote the production of pro-inflammatory cytokines and reactive oxygen species whereas other CLR affect signalling by Toll-like receptors (Geijtenbeek and Gringhuis, 2009). On the other hand, soluble CLR such as collectins including the mannose-binding lectin (MBL) and surfactants A and D, promote direct opsonization, agglutination, complement activation and phagocytosis (Hickling et al., 2004).

In addition to transmembrane receptors, there are several classes of intracellular (cytosolic) PRR, including RIG-I-like receptors (RLR) and Nod-like receptors (NLR). RLR are comprised of three members RIG-I, Mda5 and LGP2, which detect unique types of viral RNA structurally different from host RNA, allowing the discrimination between viral and self RNA (Brubaker et al., 2015). Accordingly, in the presence of viral RNA, RIG-I and Mda5 are activated and induce antiviral immunity mediated by the production of type I interferons (Brubaker et al., 2015). The NLR family encompasses over 20 members described so far in humans that sense diverse microbial structures – important for the detection of intracellular microbes – and recognizes endogenous danger signals (Davis et al., 2011; Hancock et al., 2012). NLR sensing triggers signalling pathways that lead to the production of pro-inflammatory cytokines, chemokines and recruitment of neutrophils (Davis et al., 2011; Lupfer and Kanneganti, 2013). In addition, some NLR members oligomerize to form a large protein complex termed inflammasome that induces the proteolytic cleavage and activation of caspase-1, which subsequently promotes the maturation and secretion of IL-1β and IL-18 (Davis et al., 2011; Lupfer and Kanneganti, 2013).

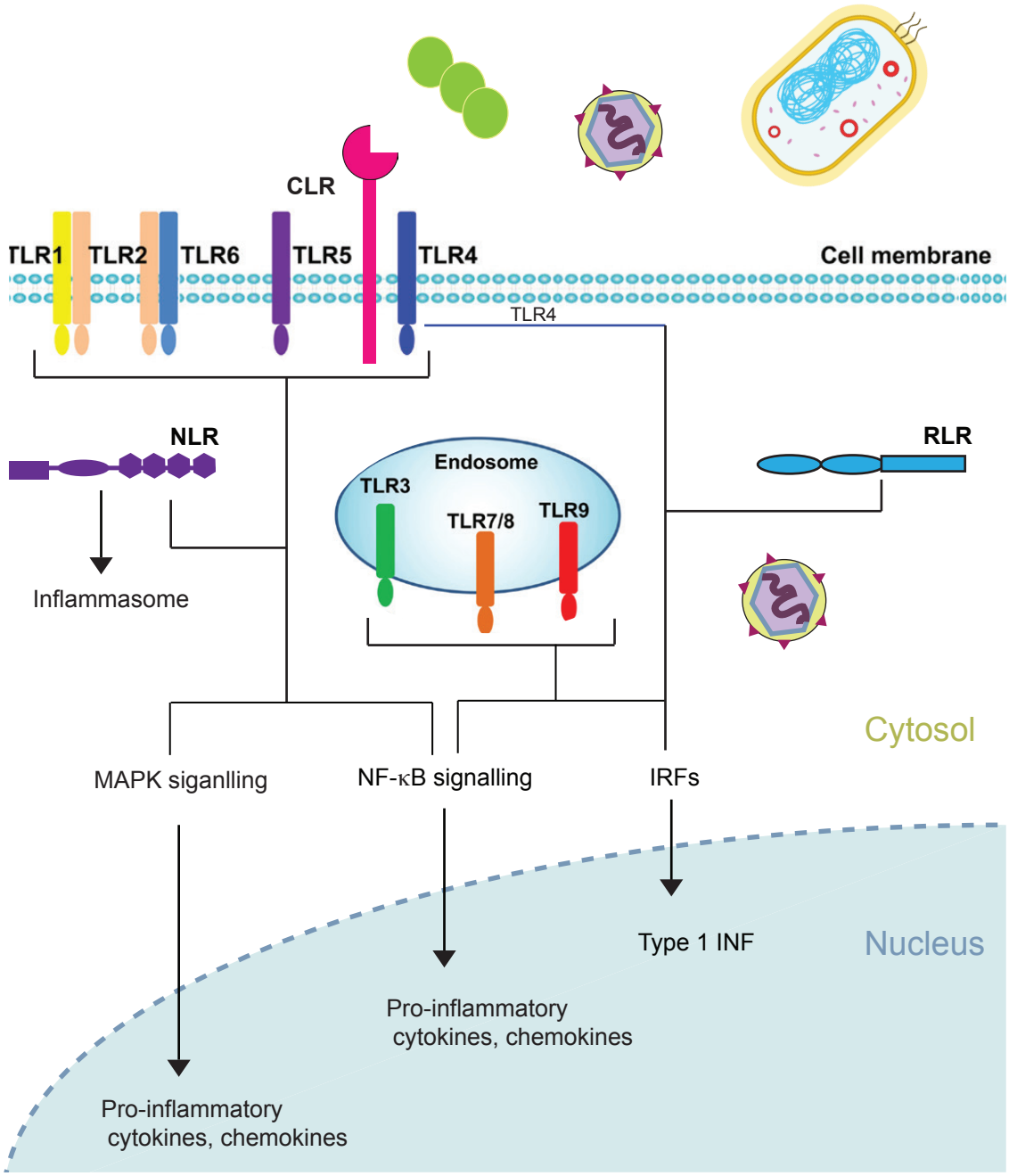

Figure 2. Schematic representation of the pattern recognition receptors (PRR) signalling pathways. PRR recognize distinct pathogen-associated molecular patterns (PAMP) and play a critical role in innate immune responses. The major PRR are Toll-like receptors (TLR); C-type lectin receptors such as dectin, RIG-I-like receptors (RLR) and Nod-like receptors (NLR). PRR signalling activate multiple intracellular pathways that lead to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling, as well as IFN-regulatory factors (IRFs). This results in the production of pro-inflammatory cytokines and chemokines, defensins and reactive oxygen species. In addition, upon stimulation by microbial and endogenous signals some NLR members oligomerize to form the caspase 1-dependent inflammasome required for the processing and secretion of IL-1β and IL-18. (Image adapted from (Zhao 2014)).

Altogether, these (and other) PRR generate a diverse repertoire of antimicrobial responses that are immediately activated for controlling infection (Kawai and Akira, 2011; Brubaker et al., 2015). However, these can be insufficient to eliminate pathogens and the adaptive immune system is often required to uphold the clearance of microbes.

1.1.2. PRR and activation of adaptive immunity

Vertebrates have evolved a sophisticated defence mechanism characterized by a high diversity of antigen receptors, antigen-derived lymphocyte clonal selection and expansion, the capacity to generate long-lasting memory cells that boost host response to a recurrent pathogenic attack, and a complex tolerance mechanism to 'self' (Mackay, 1991; Rajewsky, 1996; Nussenzweig, 1998; Pancer and Cooper, 2006).

The vast repertoire of antigen receptors in B and T cells, generated by somatic recombination, can recognize and bind specifically to an astonishing number of antigens (Flajnik and Kasahara, 2010). The generation of this great variety of receptors was unravelled through the study of antibodies in the 1970s, namely with the discovery of the variable (V), diversity (D) and joining (J) rearrangement of immunoglobulin (Ig) genes (Tonegawa, 1983; Flajnik and Kasahara, 2010). A decade later, the transmembrane T cell receptor (TCR) was discovered and found to share a similar gene domain organization and rearrangement mechanism (Davis et al., 1984; Oettinger et al., 1990; Muramatsu, 2000). The process of V(D)J recombination allows the recombination of diverse gene segments into sequences encoding functional protein chains of immunoglobulins and T-cell receptors that collectively have an extraordinary binding diversity (Flajnik and Kasahara, 2010). However, such a diverse and random antigen-receptor repertoire must be tightly controlled to prevent harmful attacks to 'self' molecules (Sakaguchi et al., 2001). Therefore, vertebrates have also evolved several immunological self-tolerance mechanisms to maintain a viable immune system (Sakaguchi, 2000). For example, during thymic selection T cells that express TCRs with high avidity for self-peptides are eliminated through TCR-agonist-induced apoptosis, whereas the ones that escape this selection are restrained by a subpopulation of mature peripheral T cells, namely CD25+CD4+ regulatory T cells (Treg) that supress self-reactive T cell activation and negatively control several immune responses (Sakaguchi, 2004; Feng et al., 2015).

Conventional B and T lymphocytes exist as naïve cells until they encounter a cognate antigen to become activated. This activation is only possible because components of

the innate system are capable, in an earlier phase, to sense pathogenic structures and present them to lymphocytes (Wykes et al., 1998; Villadangos and Schnorrer, 2007). Moreover, because adaptive immunity may require several days to develop an effective antimicrobial effect, innate immunity acts as the first line of defence (Levy and Netea, 2014).

T cells

The majority of TCRs are heterodimers consisting of an α and a β chain that recognize **antigenic fragments**, resulting from partially digested microbial agents, that are bound to the surface of major histocompatibility complex (MHC) class I or class II molecules encoded by antigen-presenting cells (APCs). Mature T cells express a complex of CD3 molecules associated with the TCR and are divided into two linages: CD8+ and CD4+ T cells that recognize peptides presented by MHC class I and class II, respectively. MHC class I molecules are expressed by all nucleated cells, and present protein fragments of cytosolic and nuclear origin, whereas MHC class II molecules are primarily expressed by professional APC, such as dendritic cells (DC), macrophages and B cells and present peptides derived from endocytic vesicles (Neefjes et al., 2011).

In addition to directly activating T cells, some APC work as **"instructors"** for the adaptive response. In this case, the microbial structure recognized by PRR will dictate the type of T cell response by establishing an association between the antigen recognized by the lymphocyte and the microbe (Fearon and Locksley, 1996; Medzhitov, 2007). Evidence suggests that this instruction is mainly provided by different DC populations that are specialized for the induction of different T cell effector responses (Iwasaki and Medzhitov, 2015). For example, DC that express RLR, NLR and TLR3 produce type 1 interferons and IL-12 in response to viruses and virus-infected cells. This in turn leads to the differentiation and activation of CD8+ cytotoxic T lymphocytes (CTL), those cells responsible for the clearance of cells presenting antigens derived from cytosolic viruses and intracellular bacteria (Iwasaki and Medzhitov, 2015). On the other hand, CD4+ T cells or 'Helper T cells' can differentiate into several types of effector cells, characterized by the production of distinct sets of cytokines (Medzhitov, 2007).

Briefly, Th1 cells are activated through the expression of IL-12 secreted by DC after the engagement of PAMP from intracellular bacteria and protozoa with TLR present on DC. Afterwards, Th1 cells secrete pro-inflammatory cytokines such as IFNγ that

activate macrophages and other cell types to induce robust antimicrobial and phagocytic responses, including the production of reactive oxygen and nitrogen species (Iwasaki and Medzhitov, 2015). Allergens and helminths indirectly activate Th2 cells differentiation, which produce a myriad of cytokines that instruct B cells to produce IgG1 and IgE, activate macrophages, recruit eosinophils and basophils, and act on epithelial cells and smooth muscle, to collectively enhance barrier defence, tissue repair and expel worms and allergens (Iwasaki and Medzhitov, 2015; Zhu, 2015). Nevertheless, a sensor for helminth detection is yet to be uncovered and differentiation of CD4+ T cells in this subtype remains elusive (though some studies correlate Th2 effector responses with a subpopulation of DC (Gao et al., 2013; Kumamoto et al., 2013). Finally, Th17 cells secrete IL-17 and IL-22 that mediate host defence against extracellular bacteria and fungi, by inducing epithelial cells to produce chemokines to recruit neutrophils and production of antimicrobial peptides (Liang et al., 2006; del Fresno et al., 2013; Segura et al., 2013). For instance, dectin-1 engagement stimulates the production of IL-6, IL-23 and IL-1β, critical for Th17 differentiation and function (Zielinski et al., 2012; del Fresno et al., 2013).

However, and despite this protective effect against microbes, under certain conditions T cells and their effector molecules are often linked to autoimmune, inflammatory and allergic diseases (Hedegaard et al., 2008; Maddur et al., 2012; Harbour et al., 2015).

B cells

The B cell receptor (BCR) is composed of a membrane bound immunoglobulin (mIg), that can recognize exposed antigenic determinants (**epitopes**) of intact molecules, such as surface proteins and carbohydrate moieties of invasive microbes (Klein, 1997). In addition, the BCR has an intracellular Igα/Igβ heterodimer responsible for signal transduction (Yuseff et al., 2013). Initially, antigen binding to BCR triggers signals that stimulate the internalization of the BCR-antigen complex; after endocytosis, the antigen is processed within the lysosome, and then the peptide fragments are presented to CD4+ T cells (Malhotra et al., 2009; Yuseff et al., 2013). This T cell–B cell cooperation provides the stimuli for the activation of B cells that differentiate into antibody-secreting plasma cells (that secrete antibodies with the same antigen binding specificity) and develop into memory B cell populations (LeBien and Tedder, 2008; Yuseff et al., 2013). The secreted antibodies neutralize pathogens through aggregation mechanisms, hamper microbe adhesion to tissues, and interfere with pathogen attachment to host

ligands. Moreover, antibodies can activate the complement cascade, which results in the lysis of pathogens or infected cells, promotes phagocytosis of antibody-coated infected cells and modulates inflammation (Forthal, 2014).

Co-signalling molecules

In addition to the antigen binding chains of TCR and BCR, other transmembrane proteins coupling to the TCR and BCR complexes are fundamental to trigger the intracellular signals required for an efficient immune response (Smith-Garvin et al., 2009). Unlike innate immune cells that can recognize microbial structures and immediately initiate a signalling pathway, T and B cells need a close physical association with APC to become activated (Bretscher and Cohn, 1970; Grakoui et al., 1999). This association is a dynamic process and involves the formation of an area of intimate membrane contact between the two cells known as the immunological synapse (IS) (Grakoui et al., 1999). During IS assembly, a functional structure of multi-molecular complexes is formed in the T cell-APC interface (Monks et al., 1998). Surface molecules include adhesion and co-signalling molecules that have a crucial role in regulating T cell activation, subset differentiation, effector function and survival. The interaction of adhesion molecules such as the lymphocyte function-associated antigen (LFA-1) on T-cells and the intercellular adhesion molecule (ICAM-1) on APC enhances the physical interaction of T cells with APC, increasing the TCR avidity for MHC/peptide (Bachmann et al., 1997). Moreover, LFA-1 contributes to a characteristic organization of the actin cytoskeleton, providing signals that elevate intracellular calcium, thereby contributing to T cell activation and MAPK pathway activation (Dustin, 2007). A similar structure is observed in B cell synapses (Carrasco et al., 2004; Yuseff et al., 2013).

Co-signalling molecules positively (co-stimulatory receptors) or negatively (co-inhibitory receptors) modulate TCR signalling. One of the best studied co-stimulatory molecule on T cells is CD28 that is constitutively expressed on the naive T cell surface where it interacts with its ligands CD80 and CD86 on the APC surface providing a key second signal for an efficient activation of T cells (Lenschow et al., 1996). Without this interaction, T cells are unresponsive to antigen presentation and no effector functions are observed. Interestingly, the CD80 and CD86 molecules are also ligands for the inhibitory protein cytotoxic T lymphocyte antigen-4 (CTLA-4), which is responsible for shutting down T cell activation (Walunas et al., 1994; Wing et al., 2008). Despite

sharing the same ligands and being structurally related, the expression profile and the ligand affinities vary remarkably between CD28 and CTLA-4. Accordingly, CD28 is constitutively expressed in T cell surfaces, whereas the surface levels of CTLA-4 are extremely low, though these can be rapidly up-regulated following T cell activation (Chambers et al., 2001). Moreover, CTLA-4 has approximately a 10-fold higher affinity for CD80 and CD86 compared to CD28 (Chambers et al., 2001). Accordingly, the induction of CTLA-4 expression upon T-cell activation works as an important mechanism to balance T cell responses and prevent a continuous inflammatory state (Saito and Yamasaki, 2003; Rudd et al., 2009). Likewise, additional co-signalling receptors are known to contribute to the outcome of an immune response – but little is known regarding their downstream signalling events and immunological functions (Chen et al., 2013).

2. The Scavenger Receptor Cysteine Rich Superfamily and the Immune System

The Scavenger Receptor Cysteine-Rich superfamily (SRCR-SF) consists of an ancient and highly conserved group of proteins that share one or more domain structures similar to the C-terminal cysteine-rich domain of the type 1 macrophage scavenger receptor (SR-A) (Freeman et al., 1990). Initially identified in the human T cell surface receptor CD5 and in the sea urchin echinoidea sperm speract receptor, SRCR domains are present in representatives from all animal phyla ranging from vertebrates (sea lamprey, avian, amphibian and mammals) to invertebrates (echinoderms, sponges and insects) (Sarrias et al., 2004b). Receptors of the SRCR-SF were shown to bind a wide range of ligands including endogenous molecules and pathogens and were proposed to play a role as PRR and to contribute to organism homeostasis mainly by regulating inflammatory signals (Sarrias et al., 2004b).

Members of the SRCR superfamily can be membrane-associated or secreted and are divided in two groups, A or B, depending on the number of cysteine residues and the resulting disulphide-bond pattern established (Resnick et al., 1994; Sarrias et al., 2004b). SRCR domains are approximately 100-110 residues long and in group A contain six cysteines that form three disulphide bonds, while in group B these have eight cysteine residues forming four disulphide bonds (Sarrias et al., 2004b). The relative position of cysteines within each group is conserved giving rise to well-defined

intradomain disulphide bonds (Resnick et al., 1996; Sarrias et al., 2004b) (Fig. 3). The SRCR domains in group A are encoded by split exons while in group B are coded by single exons (Aruffo et al., 1997; Sarrias et al., 2004b).

The crystal structure of an SRCR domain was first reported for the group A Mac-2 binding protein, which has a single SRCR domain (Fig. 3A). Structural data analysis revealed a compact fold curved six-stranded β-sheet cradling an α-helix (Hohenester et al., 1999). This structure is also valid for group B SRCR domains as confirmed by the analysis of the membrane-proximal third SRCR domain (Fig. 3B) (Rodamilans et al., 2007) and the distal SRCR domain one of CD5 (Garza-Garcia et al., 2008).

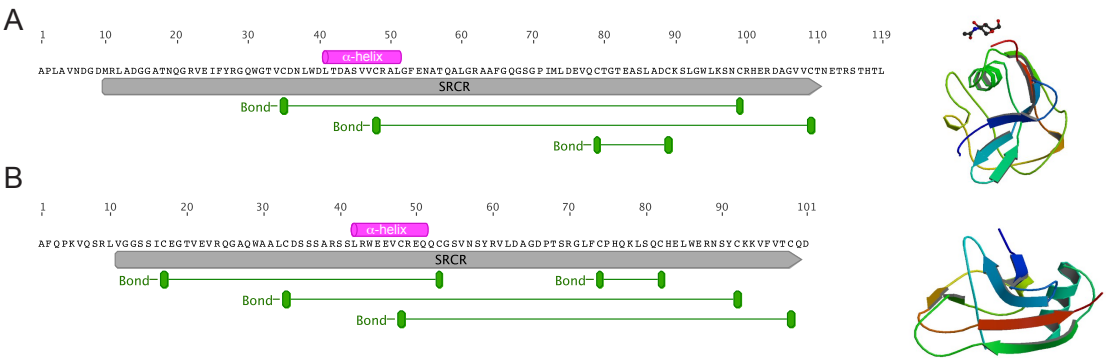

Figure 3. Arrangement of the disulphide bridges in the SRCR domains of group A (above) and B (below). Structural analysis of the Mac-2 binding protein (A) and the 3rd extracellular SRCR domain of CD5 (B) (Protein data bank ID: 1BY2 and 2OTT).

Despite the presence of the highly conserved SRCR domain, members of the SRCR-SF represent an extremely heterogeneous group that display a great functional versatility. This heterogeneity arises from subtle differences in the sequence of the SRCR domain that can affect the three-dimensional structure of each receptor (Hohenester et al., 1999), and also in the number of SRCR domains existent in the receptor, which are found singly or in tandem (up to fourteen SRCR). Also, SRCR receptors are frequently multidomain proteins, exhibiting other functional domains such as epidermal growth factor (EGF), collagen, complement C1r/C1s, Uegf, Bmp1 (CUB), zona pellucida (ZP), among others, that are involved in diverse biological roles including growth and developmental patterning, tissue repair, tumour suppression and inflammation (Martínez et al., 2011).

Nevertheless, SRCR domains are common in a wide range of proteins linked to the immune system and are thought to mediate protein-protein interactions (Hohenester et al., 1999; Bowdish and Gordon, 2009). In fact, mounting evidence strongly supports the view that SRCR proteins are relevant in diverse immune mechanisms, including

cell activation (Vilà et al., 2001; Gimferrer et al., 2004) pathogen sensing (Kang and Reid, 2003; Bikker et al., 2004; Sarrias et al., 2005; Sarrias et al., 2007; Fabriek et al., 2009; Vera et al., 2009) and inflammation (Soldevila et al., 2011; Moeller et al., 2012; Rodriguez et al., 2012; Etzerodt and Moestrup, 2013; Sanjurjo et al., 2015).

2.1. Structure and Function of SRCR-SF Group B members

To date, the human group B SRCR-SF family is found to be comprised of nine members, some with restricted expression in immune cells like CD163, M160/CD163L1, CD5, CD6 and Spα/ CD5L, whereas DMBT1, SSC4D, SSC5D and SCART1 are also found in nonimmune cells especially in the epithelia of the digestive, respiratory, and urinary tracts (Martínez et al., 2011).

Despite the increasing body of work highlighting the importance of SRCR proteins, the biological functions and ligand-binding properties of SRCR domains are poorly understood (Hohenester et al., 1999). In the following sections, we cover the current knowledge on the role of SRCR receptors in the immune system.

CD5 and CD6

Lymphocyte accessory receptors

CD5 and CD6 are close relatives within the SRCR-SF, their genes map to contiguous regions of human chromosome 11q12.2 (mouse chromosome 9) and share a similar domain organization, which is consistent with a duplication of a common ancestral gene (Lecomte et al., 1996; Bowen et al., 1997). CD5 and CD6 genes encode type 1 lymphocyte transmembrane glycoproteins of 67 kDa and 105 to 130 kDa (depending on glycosylation and phosphorylation) respectively, and contain three extracellular SRCR domains each, a transmembrane region and cytoplasmic tails well-suited for signal transduction (Hassan et al., 2004; Sarrias et al., 2004b). The structural differences between CD5 and CD6 rest on the unusual long tail of CD6 (Aruffo et al., 1997) that can give rise to five isoforms resulting from alternative splicing events (Bowen et al., 1997) and on the uncommon SRCR domain 2 of CD5 which, like group A members, has six cysteines. CD5 and CD6 are primarily expressed by thymocytes, mature T cells and the small B1a subset (Aruffo et al., 1997). In addition, CD6

expression has also been reported in various brain regions, especially in basal ganglia and cortex cerebellum (Mayer et al., 1990), as well as on NK cells (Zimmerman et al., 2006).

CD5 and CD6 physically associate with the antigen specific receptor complexes of the T and B cell receptors (Beyers et al., 1992; Lankester et al., 1994) and colocalize with the TCR/CD3 complex at the centre of the immunological synapse (Brossard et al., 2003; Gimferrer et al., 2004; Zimmerman et al., 2006). T cell activation solely through TCR signal might be ineffective resulting in a non-responsive state (anergy), in which T cells fail to proliferate and secrete cytokines in response to restimulation (Chambers et al., 1997; Smith-Garvin et al., 2009). Costimulation by other cell surface receptors, such as CD28, delivers additional signals to T cells that activate multiple effector pathways necessary for optimal T cell activation (Smith-Garvin et al., 2009). Conversely, other receptors transduce inhibitory signals that limit the stimulatory signals and negatively regulate TCR signalling leading to a downregulation of T cell responses (Chambers et al., 1997; Smith-Garvin et al., 2009). Since many of these observations hinted that CD5 and CD6 could have a functional role in T cell development and activation (Martínez et al., 2011), functional studies were undertaken to evaluate the response of CD5 and CD6 to several stimuli and differentiation stages.

Initial experiments addressing the functional activity of CD5 and CD6 relied on the use of monoclonal antibodies (mAb) and suggested a costimulatory role in lymphocyte activation for both accessory proteins regulated by TCR signalling (Gangemi et al., 1989; Bott et al., 1993; Osorio et al., 1994; Starling et al., 1996; Hassan et al., 2004). However, the generation of CD5-deficient mice ($CD5^{-/-}$) revealed that thymocytes were hyperresponsive to TCR stimulation and that signalling through the BCR leaded to apoptosis resistance and clonal expansion of B-1 cells, supporting the idea that under certain circumstances and maturation states, CD5 acts as a negative modulator of cell activation (Tarakhovsky et al., 1995; Bikah et al., 1996; Lozano et al., 2000). Interestingly, studies with transgenic mice concluded that CD5 surface expression is regulated by the intensity of the TCR signal and by TCR-ligand avidity during thymocyte selection, suggesting a fine tuning of TCR signalling by CD5 (Azzam et al., 1998; Azzam et al., 2001), in which CD5 surface levels on thymocytes and peripheral T cells parallel the avidity of their TCRs (Fulton et al., 2015; Orta-Mascaró et al., 2016). Moreover, the integral cytoplasmic domain of CD5 is required for its inhibitory function suggesting that this effect depends on intracellular effector molecules (Azzam et al., 2001).

The highly conserved cytoplasmic domain of CD5 lacks intrinsic catalytic activity but contains multiple Ser/Thr and Tyr phosphorylation sites, including two potential immunotyrosine-based inhibition motifs (ITIM) (Jones et al., 1986) that can interact with various signalling proteins, including the SH2 domain-containing phosphotyrosine phosphatase SHP-1 (Carmo et al., 1999; Perez-Villar et al., 1999), that has been implicated in the negative regulation of signalling events (Zhang et al., 2000). Conversely, the extracellular domain of CD5 was found to be dispensable for CD5-mediated inhibition of TCR signalling during thymic selection and development (Bhandoola et al., 2002). Still, and although our understanding of CD5 function and signalling mechanisms is increasing, a definite counter receptor for CD5 is yet to be discovered. Notwithstanding, several groups reported putative ligands for CD5 such as CD72 (Van de Velde and von Hoegen, 1991), gp35-40 (Biancone et al., 1996), gp150 (Calvo et al., 1999b), the framework region of IgVH (Pospisil et al., 2000), and CD5 itself (Brown and Lacey, 2010); nonetheless, a definitive and independent experimental validation of these results is required.

In contrast to CD5, CD6 has a well-known ligand, the cell surface protein activated leukocyte cell adhesion molecule (ALCAM/CD166) (Bowen et al., 1995), a widely expressed protein with five immunoglobulin superfamily (IgSF) domains found on both hematopoietic and nonhematopoietic cells (Chitteti et al., 2013). The CD6-CD166 interaction is mediated through the extracellular third (membrane proximal) SRCR domain of CD6 (CD6d3) and the N-terminal domain IgSF domain of CD166 (Whitney et al., 1995; Bowen et al., 1996). Furthermore, this interaction is critical to localize CD6 to the IS (Gimferrer et al., 2004; Castro et al., 2007).

The structure of CD6 and the binding region of CD166 have been recently resolved by X-ray crystallography (Chappell et al., 2015). Chappell and colleagues emphasized that the nonlinear domain organization of the three SRCR domains may be important to regulate the accessibility of CD166 to the membrane proximal domain of CD6 favouring the formation of CD6-CD166 heterodimers over an already described homophilic CD166-CD166 interaction (Chappell et al., 2015). Notably, the CD6-CD166 interaction was the first demonstration that SRCR domains were indeed capable to interact with proteins (Bowen et al., 1996).

The function of CD6 in TCR signalling has been a matter of intensive research. The complexity of the TCR-mediated activation of T cells and the existence of a cognate ligand limited progress on understanding CD6 function during T cell development and activation. Previous experiments using anti-CD6 mAb have suggested that CD6 could

activate T cells and enhance TCR/CD3-mediated T cell proliferation (Osorio et al., 1994; F Santos et al., 2016). In addition, the CD6-CD166 interaction was considered critical for optimal T cell activation and proliferative responses, as its disruption decreased T cell activation responses (Gimferrer et al., 2004; Hassan et al., 2004; Zimmerman et al., 2006). Considering these results, CD6 was viewed as a costimulatory molecule. However, some of the experiments were considered not to mimic the real contribution of CD6 in the observed responses and a role for CD6 remains unclear. In contrast to the proposed costimulatory role, recent findings showed that an increase on the expression levels of CD6 attenuates T cell activation by reducing calcium responses and late events such as IL-2 secretion and T cell proliferation (Oliveira et al., 2012). Moreover, the presence of CD6 in the IS as well as the interaction CD6-CD166 were not necessary for the inhibitory role of CD6 (Oliveira et al., 2012). Once again, the inhibitory regulation was dependent of the cytoplasmic domain of CD6 (as happens with CD5), but the mechanisms that lead to this inhibition are unclear.

The recent generation of mice deprived of the CD6 gene ($CD6^{-/-}$) has shed some light on the role of CD6 in T cell development and activation *in vivo* (Orta-Mascaró et al., 2016). This study showed that $CD6^{-/-}$ mice have increased numbers of immature thymocytes, implying a role for CD6 in tymocyte selection, and that immature tymocytes were hyperresponsive to TCR crosslinking (measured by intracellular Ca^{2+} flux). *In vitro* experiments established that the activation of $CD6^{-/-}$ T cells increased the expansion of peripheral T cells, especially Treg. The latter presented a lower suppressive activity when compared with Treg from $CD6^{+/+}$ mice, indicating that CD6 deficiency might hamper Treg cell effector function (Roncagalli et al., 2014; Schmidt et al., 2015; Orta-Mascaró et al., 2016). The biological significance of these results was further explored in an induced model of T cell–mediated autoimmune disease resembling rheumatoid arthritis, where $CD6^{-/-}$ mice revealed a less-favorable clinical evolution confirmed by an increase in inflammation and IL-6 and TNF secretion, when compared with $CD6^{+/+}$ mice (Orta-Mascaró et al., 2016).

This study reinforced the idea that the expression of CD5 and CD6 is, as already suspected, somehow correlated (see below). On one hand thymocytes and T cells of $CD6^{-/-}$ mice revealed a slightly reduced expression of CD5 when compared with $CD6^{+/+}$ mice, and on the other in $CD5^{-/-}$ mice the expression of CD6 in T cells was increased (Orta-Mascaró et al., 2016). These changes are worthy to further investigate as they

might provide insights regarding the regulation of both receptors (Orta-Mascaró et al., 2016).

Altogether, these results suggested that CD6 is a negative regulator of thymic and peripheral TCR-mediated signalling. Still, the precise mechanisms underlying this function are largely unexplored. One hypothesis is that CD5 may be responsible for the inhibitory role of CD6 (Oliveira et al., 2012; Orta-Mascaró et al., 2016). Interestingly, CD5 and CD6 physically associate at the surface of T cells and CD6 stimulation leads to the phosphorylation of tyrosine residues in CD5 (Castro et al., 2003; Gimferrer et al., 2003; Oliveira et al., 2012), which represents an example of function connectivity between these two receptors.

Role as PRR

Though initial studies focused on the function of membrane-bound CD5 and CD6 in lymphocyte activation, there is evidence that the ectodomains of the two proteins, containing the SRCR domains, exist as soluble glycoproteins (sCD5 and sCD6) in the serum resulting from their proteolytic cleavage of the membrane form (Calvo et al., 1999a; Ramos-Casals et al., 2001). The extracellular moieties of CD5 and CD6 share high homology with the N-terminus domain of DMBT1 and Spα, soluble glycoproteins that bind bacteria (Sarrias et al., 2004b), so sCD5 and sCD6 were proposed to have the same capacity (Sarrias et al., 2007). Indeed, this hypothesis was confirmed for sCD6, which could interact and aggregate Gram-negative and Gram-positive bacteria through LPS or LTA and PGN recognition, but not for sCD5, which failed to bind bacteria (Sarrias et al., 2007; Martínez-Florensa et al., 2013). *In vivo* experiments showed that the prophylactic administration of recombinant sCD6 improved the survival of mice in models of LPS- (Sarrias et al., 2007) and Gram-positive- (Martínez-Florensa et al., 2013) induced septic shock as well as in a lethal model of polymicrobial sepsis (Martínez-Florensa et al., 2017). rCD6 pre-treated mice exhibited low levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 in serum (Sarrias et al., 2007; Martínez-Florensa et al., 2013; Martínez-Florensa et al., 2017). Nonetheless, sCD5 was later reported to bind pathogenic and saprophytic fungi, through the cell wall component β-glucans, while sCD6 could only bind to saprophytic fungi (Vera et al., 2009). Moreover, sCD5 induced fungal cell aggregation, a mechanism that immobilizes and enhances phagocyte uptake of pathogens. Membrane-bound CD5 also binds

zymosan and this interaction mediates signalling events such as the activation of MAPK cascade and induced IL-8 cytokine release (Vera et al., 2009).

Similarly to CD6, an anti-inflammatory role for sCD5 was also predicted using a mouse model of septic shock-like syndrome induced by zymosan (Genovese et al., 2004; Vera et al., 2009). In this experiment, human recombinant ectodomain CD5 (rhCD5) was injected in mice prior to zymosan challenge, which resulted in a beneficial outcome when compared with untreated mice. The survival rate was improved, and the serum levels of IL-6 and IL-1β were reduced and so were peritoneal and liver leukocyte infiltrates. Interestingly, rhCD6 did not have a protective effect on the mice response to zymosan (Vera et al., 2009). Taken together, these studies clearly indicate that although CD5 and CD6 share similar extracellular and soluble protein structures there is a clear specificity with regard to their function in pathogen sensing. More recently, CD5 was shown to increase the susceptibility of hepatitis C virus infection of human T lymphocytes (Sarhan et al., 2012).

Spα/AIM/CD5L

Spα, also known as AIM (apoptosis inhibitory factor expressed by macrophages), Api6 (apoptosis inhibitor 6) or CD5L (CD5-like), is a secreted glycoprotein composed of three SRCR domains, expressed by tissue macrophages in the lymphoid organs including spleen, lymph nodes, thymus, bone marrow and foetal liver, and in lung epithelial cells (Gebe et al., 1997). Studies using mAbs revealed the existence of two human Spα isoforms of 38 and 40 kDa, whose difference was attributed to different sialic acid content (Sarrias et al., 2004a). Spα is a serum circulating protein with a high concentration level (2.5-10 μg/ml) that can be further increased upon inflammatory and infection conditions (Sanjurjo et al., 2015). In blood, Spα circulates associated with IgM, which protects Spα from renal excretion, contributing to the maintenance of its high levels in serum (Tissot et al., 2002; Arai et al., 2013).

Spα expression is positively regulated by the transcription nuclear factors liver X receptor/retinoid X receptor (LXR/RXR) heterodimers, which are involved in lipid homeostasis (Joseph et al., 2004), and its expression is induced in macrophages by 25-hydroxycholesterol and oxidized low-density lipoprotein (oxLDL) (Amézaga et al., 2014; Sanjurjo et al., 2015). Spα is a truly multifunctional protein involved in apoptosis, lipid metabolism, inflammation and pathogen sensing (Sanjurjo et al., 2015).

Apoptosis inhibition

The mouse homolog of Spα (~70% identity), better known as AIM (Gebe et al., 2000), was linked to an increase in the susceptibility of thymocytes to apoptosis induced by both irradiation and dexamethasone stimuli in AIM-deficient ($AIM^{-/-}$) mice (Miyazaki et al., 1999). This observation was further corroborated by experiments in which the addition of recombinant AIM (rAIM) improved the survival rate of $AIM^{(-/-)}$ purified thymocytes (Miyazaki et al., 1999). One pioneer study, using $AIM^{(-/-)}$ mice, associated the anti-apoptotic effect of AIM expression in macrophages to atherosclerosis development (Arai et al., 2005). In this condition the fatty deposits on the arterial walls are rich in macrophages that uptake oxLDL and accumulate cholesteryl esters inside cytoplasmic lipid droplets (Tontonoz et al., 1998). These high-content lipid macrophages morph into “foam cells”, which contribute to the initiation and expansion of atherosclerosis lesions (Tontonoz et al., 1998). Since the environment at these lesions is extremely proapoptotic (Geng and Libby, 2002), macrophages increase AIM expression to protect themselves from apoptosis, maintaining a state of chronic inflammation that promotes the progression of the disease (Arai et al., 2005). Accordingly, during several inflammatory conditions macrophages infiltrate into damaged tissues and increase their Spα expression to inhibit apoptosis, resulting in diseases such as chronic kidney disease, obesity-associated inflammatory diseases and liver cirrhosis (Sanjurjo et al., 2015).

Role in pathogen recognition

Spα binds and aggregates several Gram-positive and Gram-negative bacteria as well as fungi. This receptor recognizes LPS and LTA (through non-overlapping sites in humans) (Sarrias et al., 2005) and the fungal cell wall components Zymosan, mannan and β-D-glucan (Martinez et al., 2014). In the presence of Spα, monocytes stimulated with various PAMP showed a decrease in TNF-α and IL-8 secretion (Sarrias et al., 2005), which might work as a mechanism to protect tissues from local inflammation (Martinez et al., 2014). In addition, secretion of AIM by macrophages could be stimulated by the presence of bacterial and fungal PAMP (Martinez et al., 2014). *In vivo* experiments showed that mice challenged with Zymosan or LPS have decreased levels of soluble AIM, suggesting that in an early phase of infection soluble AIM might bind PAMP to help in their removal (Martinez et al., 2014). Altogether, these

observations suggest that AIM might prevent PAMP-induced sepsis and septic shock syndrome.

Interestingly, *in vitro* experiments showed that Spα upregulates the expression of antimicrobial peptides and reactive oxygen species in macrophages infected with intracellular *Mycobacterium tuberculosis*, resulting in a decrease of approximately ~70% in the number of intracellular bacteria (Sanjurjo et al., 2013). Moreover, as an anti-apoptotic protein, AIM protects macrophages against apoptosis induced by several pathogens, including *Bacillus anthracis, Escherichia coli, Salmonella typhimurium,* and *Listeria monocytogenes* (Sanjurjo et al., 2015).

Lipid metabolism

Soluble Spα typically binds to the endocytic scavenger receptor CD36, a widely expressed and multifunctional protein. In adipocytes and hepatocytes, the CD36-Spα interaction results in the internalization of Spα, which binds to and inactivates the cytoplasmic fatty acid synthase inducing a lipolytic response (Kurokawa et al., 2010). This mechanism is important to regulate cellular fat deposition in order to prevent diseases such obesity and fatty liver (Sanjurjo et al., 2015).

Spα lipid metabolism and regulation of Th17 profile

Gaublomme (2015) and Wang (2015) have shown that Spα is the functional switch that controls the function of Th17 cells. These cells display a multiplicity of functions that include the maintenance of a healthy gut mucosa (Guglani and Khader, 2010) and support the host defence against fungi (*e.g C. albicans and Aspergillus fumigatus*) and bacteria (*e.g. Staphylococcus aureus and Klebsiella pneumoniae*) and invasion (Khader et al., 2009; Kolls and Khader, 2010). However, some Th17 cells might be considered pathogenic as they relate to numerous autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, inflammatory bowel disease, allergy and asthma (Maddur et al., 2012). Surprisingly, the mechanism by which Th17 cells are converted into pathogenic cells was found to be associated with T cell lipid metabolism. Wang et al. (2015) reported that Spα was expressed preferentially in non-pathogenic Th17 cells and that its loss converts Th17 cells into “pathogenic” cells. These studies revealed that Spα

regulates Th17 cell function by regulating the fatty acid profile of T cells, which in turn modulate the activity of RORγt, the master transcription factor of Th17-cell differentiation (Wang et al., 2015).

"Soluble scavenger proteins" and biological garbage clearance

The so-called "biological garbage" is constitutively produced by our body and includes cancer cells, apoptotic or necrotic cells, degenerated cells/proteins and pathogen-invaded cells (Miyazaki and Arai, 2015). These products must be quickly cleared from our system to prevent chronic inflammation and allow normal tissue regeneration. The inflammatory environment state is mainly promoted through the release of damage-associated molecular patterns (**DAMP**) by impaired cells that activate effector scavenging cells, namely, phagocytes. However, the mechanism by which phagocytes distinguish and specifically engulf biological debris is not understood. One hypothesis is that **"marking molecules"** accumulate in garbage products and help their clearance (Miyazaki and Arai, 2015). Interestingly, Spα was pointed out as candidate (Miyazaki and Arai, 2015). Recent studies, showed that during acute kidney injury (AKI), Spα interacts with kidney injury molecule (KIM)-1, which is upregulated in injured tubular epithelial cells (TEC). KIM-1 binding to Spα promotes the efficient clearance of dead-cell debris from TEC. In the kidney, apoptotic and necrotic TEC are phagocytosed by neighbouring damaged epithelial cells, which differentiate into a phagocytic phenotype under the control of KIM-1 (Allison, 2016; Arai et al., 2016). Interestingly, Miyazaki and Arai (2015) hypothesize that a number of different proteins might be involved in garbage clearance and proposed designating these proteins as **"soluble scavenger proteins" (SSP)**. Accordingly, SSP proteins should circulate at high levels, have a "sticky nature", so they can attach efficiently to "garbage" and bind to the specific receptors that mediate engulfment through incorporation of the SSP. Excitingly, this new concept was proposed as a new therapeutic strategy, whereby SSP administration could be effective in treating refractory diseases (Miyazaki and Arai, 2015).

DMBT1/gp340/SAG

The gene **D**eleted in **M**alignant **B**rain **T**umours (DMBT1) was initially described as a tumour suppressor gene (Mollenhauer et al., 1997) for brain tumours, and later for

other epithelial cancers such as gastrointestinal and lung cancers (Somerville et al., 1998; Mori et al., 1999; Takeshita et al., 1999). Additionally, functional studies using the orthologs rabbit hensin and mouse CRP-ductin correlated DMBT1 to cell differentiation and regeneration (Ligtenberg et al., 2007). DMBT1 maps at 10q25.3-q26.1 and two secreted DMBT1 isoforms with identical amino acid sequences were also identified in bronchoalveolar fluid (gp340) (Holmskov et al., 1997) and saliva (salivary agglutinin, SAG) (Ericson and Rundegren, 1983).

DMBT1 contains between 8 and 13 tandem SRCR domains separated by SRCR interspersed domains (SID) and at the C-terminus there are two CUB domains surrounding an extra SRCR domain, followed by a ZP domain (Mollenhauer et al., 1997; Holmskov et al., 1999; Prakobphol et al., 2000). The CUB domains can be found in complement proteins such as C1s/C1r and together with ZP domains have been implicated in protein-protein interactions (Bork and Sander, 1992; Carmona et al., 2002; Kang and Reid, 2003). Glycosylation corresponds to approximately 25-40% of the total molecular weight of the protein (Oho et al., 1998), where twelve potential *N*-glycosylation sites are estimated from the DMBT1 protein sequence and a high density of *O*-glycosylation sites was proposed within the SID, which are rich in serine and threonine amino residues, resembling mucins (Bikker et al., 2002).

DMBT1 has a wide range tissue distribution, especially in epithelial surfaces, where it is highly expressed in the respiratory system, such as lung and trachea, in the gastrointestinal tract, mainly in small intestine, salivary gland and stomach, and at lower levels in brain and reproductive system (i.e. testis, uterus and mammary gland) (Holmskov et al., 1999). At the mucosal surfaces, DMBT1 is found both attached to the epithelium and secreted into the lining fluids, such as saliva, tear fluid, and respiratory mucosal secretions (Reichhardt and Meri, 2016). Additionally, in the early stage of live, DMBT1 is detected in the amniotic fluid and in the intestines of neonates, and was estimated to constitute up to 10% of the total protein amount in meconium and in the saliva of children under 3 years old, corresponding to one of the most abundant proteins in these milieus (Sonesson et al., 2011; Reichhardt et al., 2014; Reichhardt and Meri, 2016). DMBT1 is also expressed in immune cells and tissues including alveolar macrophages, peripheral blood leukocytes, spleen, thymus, lymph nodes and bone marrow (Palaniyar et al., 2010).

Role in pathogen sensing

DMBT1 was initially described as an agglutinating agent from saliva that could aggregate *Streptococcus mutans* (Ericson and Rundegren, 1983), bacteria resident in the oral cavity responsible for the formation of dental caries. The motif responsible for binding bacteria was shown to locate in the SRCR domains and consisted on the peptide sequence GRVEVLYRGSW, where five residues (xxVEVLxxxxW) were essential for bacterial binding and required to mediate bacteria agglutination in a calcium-dependent way (Bikker et al., 2002; Bikker et al., 2004). These studies showed for the first time that SRCR domains could mediate pathogen binding. Nonetheless, other SRCR members that lack this binding motif (*e.g.*, Spα), recognize bacteria as well, which indicates that other structures within SRCR domains can interact with bacterial components (Sarrias et al., 2005). DMBT1 binds a wide range of Gram-positive and Gram-negative bacteria including *E. coli*, *Lactobacillus casei*, *Helicobacter pylori, Streptococcus gordonii, S. aureus* and *Streptococcus agalactiae* (Bikker et al., 2002; Bikker et al., 2004).

DMBT1 also interacts with viruses, namely influenza A (IAV) and human immunodeficiency virus type I (HIV) and inhibits viral infection *in vitro* (Hartshorn et al., 2003; Wu et al., 2004; Hartshorn et al., 2006; Wu et al., 2006). The DMBT1-IAV interaction is not calcium dependent, and IAV binds to the sialic acid-bearing carbohydrates on the DMBT1 surface (Hartshorn et al., 2003; Hartshorn et al., 2006). Regarding HIV infection, DMBT1 interacts with the viral envelope glycoprotein gp-120 of HIV in a calcium-dependent manner disturbing HIV capacity to infect T cells in saliva (Wu et al., 2004; Wu et al., 2006). Interestingly, this inhibitory effect was shown to be mediated by the first SRCR domain of DMBT1 (Wu et al., 2006; Chu et al., 2013). On the other hand, membrane-associated DMBT1 in the vaginal epithelium facilitates transcytosis of the virus through epithelial cells (Madsen et al., 2010). This dual effect emphasizes the role of protein location and structure in biological processes including disease outcome.

Role in mucosal defence and homeostasis

Mucosal surfaces are vulnerable barriers that permit the essential contact between the organism and the environment; however, microbes or other agents can cross this delicate epithelial barrier causing infection and allergies. Therefore, these surfaces are

highly protected by specific proteins, including mucins or surfactants, tissue resident immune cells as well as commensal microbiota. DMBT1 binds several endogenous proteins such as secretory IgA, MUC5B, surfactant proteins A and D, proteins from the complement factor system, trefoil factor (TFF) and galectin-3 (Ligtenberg et al., 2007; Madsen et al., 2010; Rossez et al., 2011), to increase the eradication of bacteria and viruses (Ligtenberg et al., 2007).

Salivary DMBT1 forms complexes with IgA, which bind to a surface protein of *S. mutans*. The DMBT1-IgA interaction is mediated by the same motif within the SRCR domain responsible for bacteria binding (Ligtenberg et al., 2004). In addition, DMBT1 binds SP-D and SP-A (Holmskov et al., 1997), which are modulators of pulmonary defence; they promote microbial agglutination while regulating inflammatory responses (Gardai et al., 2003).

DMBT1 regulates the early steps of **complement activation** on mucosal surfaces (Reichhardt and Meri, 2016). Although complement is predominantly present in blood, it is also found in serous exudates on mucosal surfaces, such as in the oral cavity or the airways (Boackle, 1991; Persson et al., 1991; Reichhardt and Meri, 2016). During inflammatory and/or infectious conditions, for example upon mechanical injury, the serous exudates and blood infiltrate in the mucosal surfaces (Reichhardt and Meri, 2016), where complement components such as C1q, mannose-binding lectin (MBL), and ficolins can bind to DMBT1. These interactions activate complement cascades that lead to inflammatory responses and increase bacteria clearance (Tino and Wright, 1999; Reichhardt and Meri, 2016).

During microbial infections, a severe inflammation response may damage the mucosal epithelia; therefore, rapid regeneration strategies are important to restrain the spreading of microbial agents and the further damage of the tissue (Podolsky, 2000; Kang and Reid, 2003). Trefoil factors are small proteins that are abundantly secreted onto the mucosal surface by mucus-secreting cells and are involved in tissue homeostasis and epithelial repair upon tissue injury (Taupin and Podolsky, 2003). DMBT1 binds recombinant dimeric TFF3 in a calcium dependent manner, suggesting that this interaction may have a role in the homeostasis of mucosal surfaces (Madsen et al., 2013). Moreover, the CUB and ZP domains of DMBT1 can also interact with growth factors, cell surface molecules and cytokines (Kang and Reid, 2003) and modulate the local environment to promote mucosal defence and regeneration (Bork and Sander, 1992; Carmona et al., 2002; Kang and Reid, 2003). Interestingly, DMBT1 expression is increased in the epithelial cells of small intestine and colon in patients

diagnosed with inflammatory bowel diseases, namely ulcerative colitis (UC) and Crohn's disease (CD), and proinflammatory stimuli such as TNF-α and LPS up-regulate DMBT1 expression by epithelial intestinal cells (Renner et al., 2007; Rosenstiel et al., 2007; Madsen et al., 2013). In addition, *Dmbt1*$^{-/-}$ mice are more susceptible to dextran sulphate sodium (DSS)–induced colitis. This model of intestinal inflammation prospects that the cytotoxic effect of DSS damages the mucosa, and that the exposure of the membrane to microbial microflora will result in inflammation pointing out to a possible role for DMBT1 in intestinal mucosal protection (Renner et al., 2007; End et al., 2009). Moreover, an allelic variant of DMBT1, which lacks five SRCR exons is correlated with an increased risk for CD (Renner et al., 2007), suggesting that the SRCR domains of DMBT1 are important for its function. Another interesting observation is that the addition of recombinant DMBT1 reduces the intracellular invasion of intestinal epithelial cell lines by *Salmonella enterica,* and inhibits TLR4- and NOD2-mediated IL-8 release. This dual effect suggests that DMBT1 may limit bacterial invasion, while preventing an inflammatory outcome, which directly contributes to the maintenance of a healthy mucosa (Rosenstiel et al., 2007).

CD163

The scavenger receptor CD163 was first described as a 130-kDa glucocorticoid-regulated transmembrane glycoprotein with restricted expression in monocytes and tissue resident macrophages (Zwadlo et al., 1987; Pulford et al., 1992). Structurally, CD163 consists of nine tandem SRCR domains, a transmembrane segment and a short C-terminal cytoplasmic tail (Law et al., 1993), and is believed to descend from CD163L1 (M160), a 160-kDa protein with twelve SRCR domains that shares a similar structural organization (Grønlund et al., 2000). Alternative splicing of CD163 results in three isoforms with different cytoplasmic tail sizes (Law et al., 1993; Ritter et al., 1999; Schaer et al., 2006), with all splice variants containing phosphorylation target sequences for creatine kinase and protein kinase C (Van Gorp et al., 2010a); the isoform with the shortest tail is the most abundant. In addition to the membrane-bound CD163, a soluble version of CD163 (sCD163) can be found in plasma (~1-3 mg/liter) (Møller et al., 2002). During sepsis or inflammatory conditions that affect macrophage activity, the levels of sCD163 in serum might raise many-fold (Madsen et al., 2004; GaÏNi et al., 2006; Weaver et al., 2006; Etzerodt et al., 2010; Etzerodt and Moestrup, 2013). The molecular weight of sCD163 is equivalent to the ectodomain of surface

CD163 pointing to a complete shedding of the extracellular domain (Møller et al., 2002), which arises from the cleavage of the cell surface CD163 by the enzymes ADAM17 and ADAM10 (Etzerodt et al., 2010; Kneidl et al., 2012).

Clearance of the Hp-Hb complex

CD163 is a multifunctional protein best-known for its role in the clearance of the complex of haptoglobin (Hp) and haemoglobin (Hb) formed after the lysis of red blood cells (Kristiansen et al., 2001). The removal of haemoglobin by CD163 constitutes an important defence mechanism as it prevents the toxic effect of the heme molecule. During intravascular haemolysis, haemoglobin from erythrocytes is released into circulation where it binds haptoglobin forming the Hp-Hb complex (Graversen and Moestrup, 2015). The Hp-Hb complex interacts with surface CD163, through a region involving the second and third SRCR domain with extremely high affinity and in a calcium-dependent manner (Madsen et al., 2004). The complex is endocytosed by macrophages and the heme molecule is degraded by hemeoxygenases into biliverdin/bilirubin, carbon monoxide (CO) (Ryter et al., 2007) and iron. Biliverdin/bilirubin are potent antioxidants whereas CO exerts anti-inflammatory responses (Philippidis et al., 2004; Couper et al., 2008; Soares and Bach, 2009; Martínez et al., 2011; Maddur et al., 2012). Furthermore, the binding of CD163 to Hp-Hb complexes triggers cytokine secretion, especially IL-10, which limits T cell activation and proliferation as well as the production of proinflammatory cytokines (Philippidis et al., 2004; Couper et al., 2008; Martínez et al., 2011; Maddur et al., 2012). IL-10 release in turn up-regulates both CD163 and hemeoxygenase-1, thus further potentiating Hb uptake, creating a positive loop (Graversen and Moestrup, 2015). This proficient mechanism prevents tissue damage resultant from the toxic and oxidative heme molecule and might help to control infection by reducing iron availability to haemolytic bacteria and trypanosomes (Weaver et al., 2006; Vanhollebeke et al., 2008; Graversen and Moestrup, 2015).

CD163 has also been identified as an erythroblast adhesion receptor, which promotes the growth and/or survival of erythroblasts during erythropoiesis (Fabriek et al., 2007) and is scavenger receptor for the cytokine TNF-like weak inducer of apoptosis (TWEAK) (Bover et al., 2007; Moreno et al., 2009).

Role in pathogen sensing

Amongst all the members of the SRCR-SF group B members, CD163 is the only surface receptor with restricted expression on monocytes/macrophages. Fabriek and colleagues first identified human CD163 as a macrophage surface receptor for recognition of intact Gram-positive and Gram-negative bacteria, namely *S. mutans* and *E. coli*, respectively (Fabriek et al., 2009). The authors supported the idea that bacterial recognition by CD163 generates signals that trigger macrophage cytokine production including TNFα, IL-1α and IL-6, all pro-inflammatory cytokines that could mediate a local immune response, but excluded a role for CD163 in bacteria phagocytosis. CD163 interaction with bacteria was mapped to the second SRCR domain, which included the binding motif xxVEVLxxxxW. Supporting a pathogen-sensing role for CD163, a subsequent study showed that in the presence of *S. aureus,* CD163 is shed from monocytic membranes and binds to the host fibronectin (FN) bound to the bacteria surface fibronectin-binding proteins (FnBP) (Kneidl et al., 2012). This event enhances the phagocytosis of bacteria and activates endothelial infected cells, reducing bacteria viability inside these cells. Indeed, this is a highly sophisticated mechanism to reduce bacteria spreading as the adhesion of *S. aureus* to FN is essential for cell infection (Kneidl et al., 2012). Also, CD163 was shown to bind several types and strains of bacteria with different affinities, for example it bounds *S. aureus* and *Streptococcus pyogenes,* but not *Staphylococcus epidermidis, E. coli,* or *Streptococcus agalactiae* (Kneidl et al., 2012).

During coevolution of host-microbe interactions, pathogens have evolved mechanisms to exploit host surface receptors to invade cells so they can replicate and/or escape from immune surveillance (Van Gorp et al., 2010a). CD163 is one of such receptors In pigs, the African swine fever virus (ASFV) and the porcine reproductive and respiratory syndrome virus (PRRSV) have been shown to enter into monocytes and macrophages through CD163 (Sanchez-Torres et al., 2003; Van Gorp et al., 2009; Van Gorp et al., 2010a; Van Gorp et al., 2010b). In the PRRSV infection, the fifth SRCR of CD163 is indispensable for viral infection (Van Gorp et al., 2010b; Ma et al., 2016). In addition, the levels of CD163-expressing macrophages are upregulated in patients with HIV (Fischer-Smith et al., 2008), and the rate of HIV infection is enhanced in macrophages that express high-levels of CD163 (Tuluc et al., 2014). Moreover, upon HIV infection, CD163 is shed from the monocytes membrane into the plasma and is positively correlated with the pathogenesis of HIV infection (Burdo et al., 2011).

CD163L1/M160

As previously mentioned, CD163L1 shares a high structural similarity with CD163, with the two genes mapped at chromosome 12p13.3 and next to each other, compatible with a gene duplication event (Grønlund et al., 2000; Moeller et al., 2012). The domain organization of CD163L1 is composed of a large extracellular region containing twelve SRCR domains, followed by a transmembrane region and a cytoplasmic tail with two alternative isoforms (Grønlund et al., 2000; Moeller et al., 2012). The tissue distribution profile of CD163L1 is similar to CD163 in the lymphoid and intestinal tissues as well as in the chorionic placental villi resident macrophages (Moeller et al., 2012). However, CD163L1 is absent or weakly expressed in long-lived macrophages with self-renewal properties such as the Kupffer cells from liver, alveolar macrophages from lung and cells from cerebellum in opposition to CD163. Moreover, CD163L1 is not capable of Hp-Hb complex endocytosis and preliminary data indicate that CD163L1 lacks the ability to bind bacteria, pointing out that CD163 and CD163L1 are functionally distinct. Nevertheless, though a ligand for CD163L1 remains to be identified, CD163L1 has endocytic properties and its expression is also regulated by cytokines; for instance, pro-inflammatory stimuli such as LPS, TNFα and IFNγ downregulate CD163L1, while IL-10 upregulates CD163L1, leading to an immunomodulatory/anti-inflammatory macrophage phenotype (Moeller et al., 2012).

Role in inflammatory bowel diseases

Resident macrophages in the intestinal mucosa derive from blood monocytes that differentiate in the gut mucosa influenced by local environment factors (Bain et al., 2013; Bain and Mowat, 2014). In healthy individuals, macrophages are stimulated by the immunosuppressive/anti-inflammatory cytokine IL-10, to promote a “tolerant” environment to commensal gut microbiota while protecting intestine epithelial monolayer against pathogens (Bain and Mowat, 2014). One subset of protective macrophages, highly abundant in the colon of heathy individuals, are resident macrophages expressing CD163L1 that produce IL-10. However, in severe inflammatory conditions, other subsets of macrophages, namely CLEC5A+ macrophages, rise their levels and are stimulated by pro-inflammatory cytokines engaging in aberrant immune responses towards commensal gut bacteria. Interestingly, in inflammatory bowel disease the expression of IL-10 by resident

CD163L1+ macrophages is somehow lost and these cells are unresponsive to pro-inflammatory stimuli (González-Domínguez et al., 2015).

SCART1

A recent cloned member of the human group B SRCR-SF is SCART1, a gene that is mapped to chromosome 10 and encodes three splice variants containing 2, 4, or 5 SRCR domains (Holm et al., 2013). In addition, one other transcript displaying a transmembrane domain was detected but remains to be cloned. This observation suggests that, like its mouse homologue (mSCART1), hSCART1 may exist as a membrane-bound receptor (Holm et al., 2009; Holm et al., 2013). Two of the cloned hSCART1 transcripts encode a cytoplasmic tail, which contains several potential phosphorylation and internalization motifs and that like CD5 and CD6, may be involved in signal transduction (Holm et al., 2009; Holm et al., 2013). Quantitative real-time PCR analysis showed that hSCART1 is mainly expressed on T lymphocytes and analysis of tissue distribution showed highest expression levels in the intestinal anatomical barrier (Holm et al., 2013). Interestingly, immunohistochemistry (with a peptide sequence unique for hSCART1) showed a prominent staining of the brush borders of placental villi resembling the high expression of SSC5D in placenta (Gonçalves et al., 2009; Holm et al., 2013). Altogether, the hSCART1 expression prolife suggests a role in immune defence (Holm et al., 2013). Nonetheless, the function of hSCART1 remains to be clarified. Despite initial studies having indicated that mSCART1 was not able to bind to a selection of PAMP and bacteria (Fink et al., 2010), it cannot be ruled out that mSCART1 or hSCART1 might bind to other microorganisms such fungi, mycobacteria, or parasites and behave as PRR (Fink et al., 2010; Holm et al., 2013).

SSC4D

Human SSC4D/S4D-SRCRB is a soluble member of the SRCR-SF composed of four SRCR domains separated by Pro-, Ser- and Thr-rich polypeptides. Northern blot analysis showed that SSC4D is expressed as two major mRNA species: one of 2.8 kb, with a restricted tissue expression pattern (mainly kidney and placenta) and having a predicted molecular mass (Mr) of 55,600, and the other of 1.5 kb, with a broader distribution (Padilla et al., 2002). Recombinant SSC4D was reported to bind several

microorganisms, including Gram-positive and Gram-negative bacteria, through the recognition of LTA or LPS, respectively, and fungi (Miró et al., 2012). However, its function remains unclear.

SSC5D

Studies carried out in our laboratory revealed the existence of a new member of the SRCR-SF. The **S**oluble **Sc**avenger with **5** SRCR **D**omains (SSC5D) receptor is encoded by a gene located in chromosome 19q13.4 and is composed of 14 exons that code for a 1573-aa polypeptide with a predicted molecular mass of 165.7 kDa, details of the genomic organization are shown in Fig 4. (Gonçalves et al., 2009).

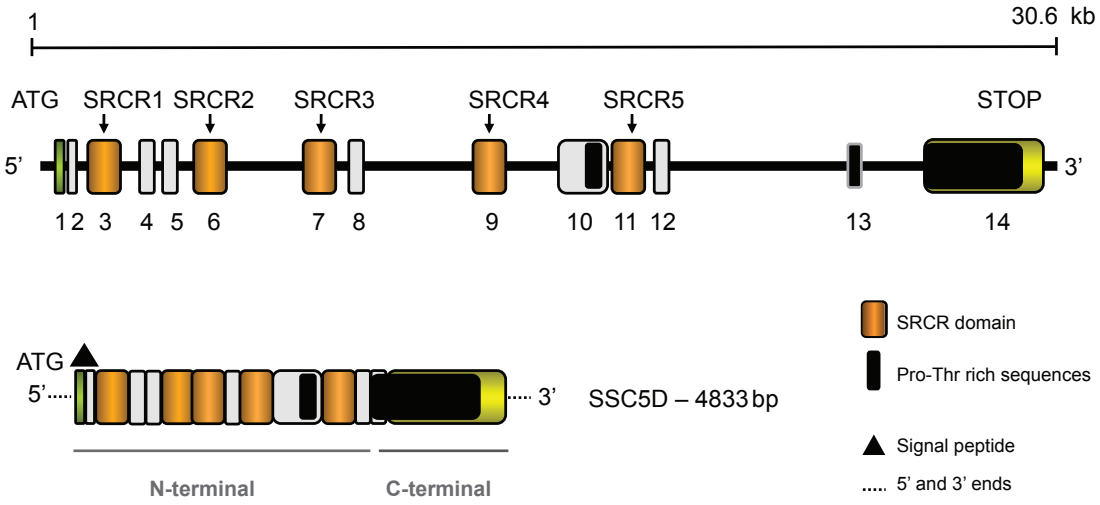

Figure 4. Genomic organization of the SSC5D gene. The gene spans over 30.6 kb in chromosome 19q13.4. Coding regions are represented as boxes, and introns as connecting lines. SRCR domains are in orange, internal exons in grey, the coding regions containing the stop codons are in yellow, and that containing the start codon is in green. Black areas represent proline and threonine-rich sequences. The localization of the signal peptidase cleavage is indicated by a black triangle (Gonçalves et al., 2009).

Full-length SSC5D consists of two structurally distinct parts: an N-terminal domain (exons 1-12), which contains five SRCR domains, and a C-terminal domain (exons 13-14) composed of a high number of repetitive sequences rich in Pro and Thr residues, *e.g.*, PDPTTT or PHPTTT, reminiscent of similar sequences present in **mucins** (Gonçalves et al., 2009)**. Mucins** are large glycoproteins with many *O*-linked glycans that are found in mucus, where they are responsible for the protection of epithelial surfaces from dehydration, mechanical injury and pathogens (Perez-Vilar and Hill, 1999). In addition, this post-translational modification helps proteins to maintain an

extended conformation, producing a long filamentous structure, where *O*-glyans can be packed very tightly (Dekker et al., 2002). The SSC5D **mucin-like domain** is putatively highly *O*-glycosylated, with 144 potential *O*-glycosylation sites (Fig. 5), which can contribute for its biological function. Indeed, *O*-glycans are important in a wide range of biological processes, including immune responses, where they can that act as ligands for receptors that mediate host-pathogen interactions (Varki, 1993).

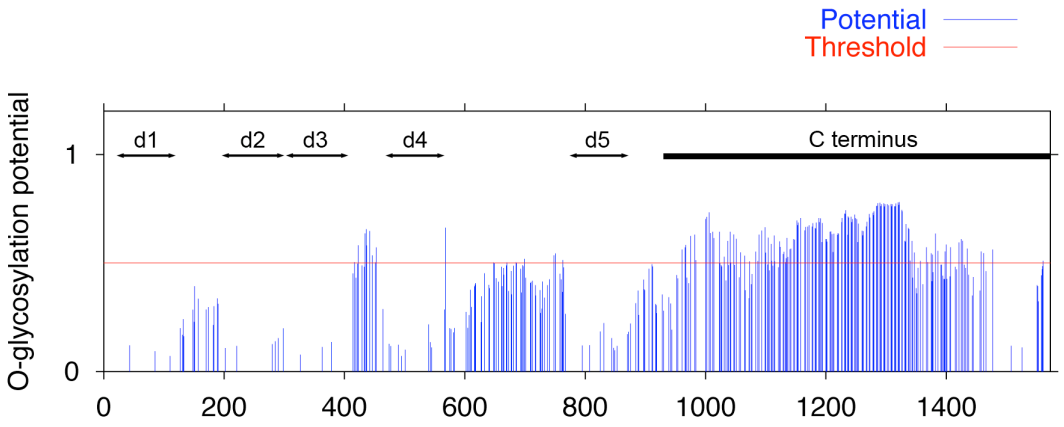

Figure 5. Predicted O-glycosylation of SSC5D using the NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc-3.1/). The C-terminal region of SSC5D, which includes sequences encoded by exons 13 and 14, is putatively extensively glycosylated, with 144 potential O-glycosylation sites in a 476-long amino acid stretch, i.e. approximately 30% of amino acids with a glycosylation potential above the calculated threshold (Gonçalves et al., 2009).

Northern blotting analysis of SSC5D revealed an abundant mRNA species of 4.8 kb in **placenta** and **spleen**, and at lower levels in **colon** and **lung** (Gonçalves et al., 2009). In addition, a transcript of 3 kb was also observed in placenta and spleen, probably corresponding to an alternative spliced variant. Moreover, qPCR analysis of SSC5D revealed its expression in immune cells such as monocytes, CD4+ T cells and at lower levels in CD8+ cells. Interestingly, soluble SSC5D was found to be upregulated in the synovial fluid of osteoarthritis patients (Balakrishnan et al., 2014), which insinuates a role in inflammation. Altogether, the peculiar characteristics of SSC5D suggest a role in immune defence, tissue homeostasis and foetal development.

In recent studies, the mouse homologue of SSC5D (S5D-SRCRB/Ssc5d) was characterized as a secreted glycoprotein exclusively expressed in the genitourinary and digestive tract that was able to interact and aggregate bacteria and fungi (Miró-Julià et al., 2011). Also, S5D-SRCRB binds bacterial and fungal cell wall components

and its presence was shown to inhibit IL-8 secretion induced by a cell line expressing TLR2 and exposed to PGN (Miró-Julià et al., 2011). Moreover, in a study using a mouse model of urinary tract infection, the mRNA levels of S5D-SRCRB in kidneys were increased after bacterial aggression, suggesting that S5D-SRCRB may have a protective role in the urinary system (Miró-Julià et al., 2014).

Like other members of the SRCR-SF, S5D-SRCRB binds endogenous extracellular matrix components including laminin, galectin-1 and galectin-3 (Miró-Julià et al., 2014). Galectin-3 interacts with S5D-SRCRB through its carbohydrate-binding region, which suggests that S5D-SRCRB glycosylation pattern may be important for this interaction. This ubiquitously expressed lectin recognizes β-galactoside structures and is involved in various biological processes, including cell development and immune reactions (Dumic et al., 2006). However, the significance of the S5D-SRCRB-galectin 3 interaction is yet to be explored.

Nevertheless, information about human SSC5D is scarce and there can exist interspecies differences in the function of proteins. For example, the mouse orthologue of CD163, which shares 70% of amino acid similarity is resistant to endotoxin- and phorbol ester-induced shedding (Etzerodt et al., 2014). Therefore, a deeper study to understand this peculiar human SRCR member is required to provide insights into the biological function of SSC5D.

3. Molecular approaches to study PRR-pathogen interactions

During the last years, there is an increased interest in the field of PRR research and their ligands (Kumagai and Akira, 2010; Levy and Netea, 2014). Innate immunity is no longer considered a ‘non-specific’ event, as several PRR have shown to specifically interact with well-defined structures (Akira et al., 2006; Levy and Netea, 2014). As already mentioned, innate components shape adaptive responses to efficiently clear pathogens. Moreover, with the recent discovery of the enhanced ability of innate immunity to respond to secondary infection, the study of novel vaccine formulations is expanding (Levy and Netea, 2014). This capacity is known as trained immunity (modulated by epigenetic reprograming) and is defined as an enhanced response to a reinfection (by the same microorganism) that may also provide cross-protection to different pathogens (Netea et al., 2011). This memory-like mechanism is independent

of B and T cells, and involves macrophages and NK cells. Trained immunity builds on phenotypic changes in innate immune cells that probably involves differential expression of PRR, differences in the monocyte/macrophage cell subpopulations and/or different functional responses (Netea et al., 2011).

In addition, several PRR, including some SRCR members, were shown to have a therapeutic value in animal models of diseases, and are potential markers for the diagnosis and pathogenesis of several conditions (Martínez et al., 2011). As mentioned earlier, SRCR receptors bind several pathogens and microbial structures; still, the list of new molecules binding SRCR proteins continues to expand. However, the components on microbe surfaces and their secreted virulence factors present a great structural diversity that pose some challenges in the analyses of complex interactions. Therefore, to gain a better insight into specific interactions it is important to use not only conventional molecular biology techniques, but also to develop more sensitive approaches (Kumagai and Akira, 2010) to elucidate PRR-pathogens interactions.

Surface plasmon resonance (SPR)-based biosensors have emerged as a promising technique to detect biomolecular interactions (Piliarik et al., 2009; Singh, 2016) (details of this method are described in chapter 2). Briefly, in an SPR analysis, a ligand (biorecognition element) is immobilized on a sensor chip, then its putative binding partners (analytes) are flowed along the sensor surface, where specific interactions are transduced into measurable signals (Homola, 2008). SPR–based biosensors can be used to detect interactions with whole bacteria cells, usually by using specific and high affinity antibodies (Byrne et al., 2009). However, besides antibodies researchers are also exploring new ligands to detect whole cell bacteria (Ahmed et al., 2014; Templier et al., 2016). Some of these ligands not so commonly used include host proteins. Fibronectin is a host matrix protein that is a common target for bacteria, including *S. aureus and Staphylococcus epidermidis* (Vaudaux et al., 1993; Foster and Höök, 1998; Francois et al., 1999; Williams et al., 2002). In one pioneer study, SPR biosensors were capable of detecting the binding of *S. aureus* to fibronectin, and a much lower affinity interaction of fibronectin with *S. epidermidis* (Holmes et al., 1997). In addition, using different fibronectin fragments, the authors observed that the *S. aureus* and *S. epidermidis* interacted with fibronectin through different binding sites. This observation was later confirmed by additional studies using a shotgun phage display cloning tool that unveiled a new fibronectin-binding protein for *S. epidermidis* (Williams et al., 2002). Intriguingly, we found a single study using a PRR, namely DMBT1, to perceive interactions with bacteria (Oli et al., 2006). In this study, DMBT1 was shown to bind *S.*

mutans but not a *S. mutans* mutant (PC3370) that lacks the bacterial P1 antigen, which is presumed to interact with DMBT1 (Demuth and Irvine, 2002).

Surface plasmon resonance (SPR), has been successfully used for the rapid detection of pathogens and presents several advantages when compared with conventional studies. SPR approaches allow the rapid detection of interactions without labelling steps of both proteins and bacteria, is more sensitive, and one can observe the binding events in real-time (Lundström, 1994; Dudak and Boyacı, 2009). Moreover, it is also less time consuming and not so laborious, comparing with conventional techniques, such as immunoprecipitation assays, agglutination tests or flow cytometry (Lundström, 1994; Boozer et al., 2006; Dudak and Boyacı, 2009; Wilson et al., 2011). It usually requires less reagents, and one major advantage is that by using a multi-channel device, one can perform simultaneously various analyses (Boozer et al., 2006).

These advantages make SPR biosensors strong candidates for investigating PRR interactions with whole bacteria.

4. Aims

Our laboratory has been interested in studying the function of the SRCR-SF members upon immune challenges. One important achievement was the cloning of a new SRCR glycoprotein named SSC5D. The predicted structural characteristics of SSC5D hint at a role as an innate immune receptor. Also, initial studies showed the expression of SSC5D transcripts in mucosal tissues and in placenta. In these locations, complex immune interactions are responsible not only for protecting the host and the developing foetus against the potential harmful effects of pathogens, but also to control exacerbated immune responses. Therefore, the characterization of a new protein potentially relevant in these milieus holds the prospect of furthering our knowledge of immune events at these interfaces.

Accordingly, this work aims to

1) determine the binding specificities of the scavenger receptor domains, and of the C-terminal domain of SSC5D,

2) explore the ability of SSC5D to bind to different strains of bacteria and

3) to comprehend the biological relevance of SSC5D by performing an extensive characterization of the expression of SSC5D in several tissues and by initiating the generation of a mouse strain with a disrupted *Ssc5d* gene.

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Chapter 1

Production of the N- and C-terminal domains of SSC5D; screening for cell surface ligands

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1.1. Introduction

It is now appreciated that the multitude of functions exhibited by SRCR-SF members are indispensable for the organism homeostasis. Although a precise biological function of SRCR domains has not yet been clearly established, several lines of evidence suggest that these domains are important for mediating protein-protein interactions (Martínez et al., 2011). These are vital for many cellular processes and their affinities and specificities are finely tuned to the functions they undertake (Chen et al., 2013). Since the biological role of a protein is also defined by its interactions in the cell, the discovery and characterization of potential ligands and interaction partners for SSC5D would, on, one hand help to elucidate its function, and one the other contribute for the understanding the biological significance of SRCR domains.

SSC5D is found in several tissues and circulates at high levels in the bloodstream, where it can potentially interact with a variety of cells and molecules. Therefore, it is conceivable that specific interactions between SSC5D and cell surface receptors may occur. The soluble SRCR protein Spα, which is also highly present in the serum, was reported to interact with several cells. Flow cytometric analysis of the interaction of recombinant Spα with several immune cell types showed that Spα bound to myeloid cell lines and lymphocyte-derived cell lines as well as to peripheral blood monocytes (Gebe et al., 1997; Sarrias et al., 2004). However, the ligand or ligands responsible for the aforementioned interactions were not identified.

In fact, the T cell surface receptor CD6 is the only SRCR member having a well-stablished cell surface ligand, ALCAM/CD166 (Bowen et al., 1995), a protein expressed by various cell types. Interestingly, the CD6-CD166 interaction is relatively strong with a dissociation constant (K_d) of ~0.4–1.0 μM (Hassan et al., 2004), so the identification of the CD6 ligand probably benefited from this resistant physical association. Importantly, a second ligand was reported to interact with CD6 (Joo et al., 2000; Saifullah et al., 2004) suggesting that SRCR proteins may have multiple ligands. On the contrary, attempts to identify ligands for CD5, the closest relative of CD6 were unsuccessful. Despite several surface binding partners for CD5 being reported, namely CD72 (Van de Velde and von Hoegen, 1991), gp40-80 (Biancone et al., 1996), gp150 (Calvo et al., 1999) and even CD5 itself, all results lacked confirmation by independent studies (Brown and Lacey, 2010; Santos, 2012). Although much effort has been devoted to find cell surface receptors for SRCR, especially for membrane-bound receptors, the immense complexity of such interactions poses a real challenge.

SSC5D is a 1573 amino acid-long glycoprotein with a predicted molecular mass of approximately 165 kDa (Gonçalves et al., 2009). SSC5D can be divided into two well-defined fragments: an N-terminal domain (N-SSC5D), which contains the five SRCR domains, and the C-terminal domain (C-SSC5D), a mucin-like domain due to the high number of repetitive sequences rich in proline and threonine residues. These residues are putatively heavily *O*-glycosylated, a typical feature of mucins that are known for their high carbohydrate content (Julenius et al., 2005). For the purpose of investigating putative interactions of SSC5D and given the distinctive characteristics of the N- and C-SSC5D moieties, we decided to produce and characterize each domain independently. Furthermore, if a positive interaction between SSC5D and a cell is observed, it can be assigned to either the N- or C-terminal SSC5D domains.

However, cell surface molecules interact with generally low affinities, with K_d between 1 and 100 μM (Davis et al., 2003). Therefore, it is expected that SSC5D interactions with cell surface receptors may have a short lifetime and be transient, meaning that putative complexes are dynamic and may dissociate rapidly (Nooren and Thornton, 2003). As this particularity may hamper the identification of cells that could express a ligand for SSC5D, we used an approach designed to improve the binding of SSC5D to eventual cell surface receptors. This experimental method consisted in producing our receptors in tetrameric form to increase the avidity of receptors for its ligands, and analyse interactions using flow cytometry. Multimeric forms are used to increase the sensitivity of detection and were initially designed to identify low affinity interactions (Altman et al., 1996; Ogg and McMichael, 1998), and given that in our system tetrameric avidin is coupled to fluorescent dye, binding of the ligands to cells can easily be followed in flow cytometry. This research protocol builds on previous experimental designs developed by our group to identify putative ligands for CD5 (Santos, 2012). In the event of finding cells that interact with SSC5D, a subsequent approach using genomic sequencing combined with bioinformatics tools will be applied to find putative ligands in the interacting cells (Santos, 2012).

1.2. Materials and Methods

Cloning of the N-terminal (N-SSC5D) and C-terminal (C-SSC5D) domains of SSC5D

First strand cDNA was synthesized using 1 µg of total placenta RNA, 0.5 µg oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. To amplify the N-terminal domain (N-SSC5D) containing the 5 SRCR domains (exons 1-12), first a nested polymerase chain reaction (PCR) was performed using the sense primer 5'-TATAATGGATCCGAGCGCCTGCGCCTGGCCGAT-3´ and anti-sense 5'-AATAGGATCCCTCTTGTGTCCGGCAGGCGCCTTATTGCTGG-3' (*Bam*HI site underlined), in a 50 µl mixture containing 1 µM of each primer, 100 ng of placenta cDNA, 10 mM dNTPs, 0.5 µl DMSO and 1 U of phusion enzyme (FINNZYMES) with the following cycling conditions: 98 ^{o}C (60 s) and 25 cycles of 98 ^{o}C (15 s) and 72 ^{o}C (90 s), followed by a final extension of 72 ºC for 5 min. The amplified sequence was subsequently purified with the QIAquick PCR purification kit (Qiagen), digested with *Bam*HI and cloned into the *Bam*HI/*Bcl*I–digested pEE14-BirA vector, a lab modified version of the pEE14 vector (Lonza biologics), using the T4 DNA Ligase (Roche). The ligation products were transformed into TOP10 *E. coli*-competent cells (Invitrogen) and the resulting clones were checked by sequencing. The final construct contained chimeric cDNAs encoding, in the following order, a signal peptide, a hemagglutinin (HA)-tag, N-SSC5D, a BirA recognition sequence and a 6 × histidine (His)-tag.

The C-terminal domain (C-SSC5D), which contains exons 13 and 14, was amplified from placenta cDNA by PCR with the sense primer 5'-AACCCTCTACTAGGTCTAGATTCTACAGGCAGCAAAGATGGTTACAAGCTT- 3' and anti-sense 5'-TAAGATATCTCACACGTCTCCCCTC-3' in a mixture similar to the aforementioned using different cycling conditions: 98 ºC (30 s) and 25 cycles of 98 ºC (10 s), 60 ºC (30 s) and 72 ºC (90 s), followed by a final extension of 72 ºC (7 min). The DNA product was purified and cloned into the pGEM-T vector (Promega). After bacterial transformation a single colony was picked (miniprep 19), purified and sequenced with the universal primer SP6 and T7. A second PCR for adding the *Bam*HI restriction sites was performed with the sense 5' – CATGCAGGATCCGGCAGCAAAGATGGTTACAAG 3' and anti-sense ATGTATCGGATCCCACGTCTCCCCTCAGG primers in the specified mixture: 1 µM of each primer, 200 ng of miniprep 19, 10 mM dNTPs and 2.5 U pwo polimerase (Roche) in a final volume of 50 µl. The temperature profile was: 95 ºC (2 min) and 30 cycles of 95 ºC (30 s), 60 ºC (30 s) and 72 ºC (4 min), followed by a final extension of 72 ºC for 7 min. The purified C-terminal domain sequence (C-SSC5D) was inserted into the *Bam*HI and *Bcl*I sites of the pEE14-BirA vector in order to obtain a C-SSC5D construct equivalent to the one described for N-SSC5D.

Cloning of Spα

Human Spα was amplified from spleen cDNA by nested PCR using two sets of different primers, one localized in an external region of the gene and a second containing the restriction sites to insert the Spα sequence into the pEE14-BirA vector. The first PCR was performed in a 50 µl mixture including 1 µM of the sense primer 5'-TGGCTCTGCTATTTCTCCTTG and the anti-sense 5' – AGGTCAAGCAACACCAGGATA, 100 ng of spleen cDNA, 10 mM dNTPs, 1.5 µl DMSO and 1 U of phusion enzyme (FINNZYMES) with the following cycling conditions: 98 ºC for 30 s, 35 cycles of 98 ºC for 10 s, 62 ºC for 30 s and 72 ºC for 1 min, followed by a final extension of 72 ºC for 10 min. The amplified product was purified using GeneClean kit (MP Biomedicals) and 5 µl of this amplicon was used as a template to perform a second PCR using the sense primer 5' – TTAGGATCCTCTCCATCTGGTGTGCGGCTG and the anti-sense 5' – CAAGGATCCACCTGAGCAGATGACAGCCAC (*Bam*HI site underlined) with the PCR conditions described previously. The amplified sequence was subsequently digested with *Bam*HI and cloned into the *Bam*HI/*Bcl*I–digested pEE14-BirA vector, using the T4 DNA Ligase (Roche). The ligation products were transformed into TOP10 *E. coli*-competent cells (Invitrogen) and the resulting clones were checked by sequencing. The final construct contained chimeric cDNAs encoding, in the following order, a signal peptide, a HA-tag, Spα, a BirA recognition sequence and a 6 × His-tag.

Cell lines

Cell lines JTAg E6.1, K562 and THP-1 were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% dialyzed foetal calf serum (FCS) (First Link), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a 5% CO_2 humidified incubator. RPMI 1640 and all supplements were obtained from (Gibco, Life Technologies) except for FCS (First Link). Cell lines Caco-2, HeLa, HCT-116, JEG-3, HEK 293 and CHO-K1 were maintained in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a 5% CO_2 humidified incubator. DMEM and all supplements were obtained from (Gibco, Life Technologies) except for FCS (First Link).

Transient protein expression in HEK 293 cells

HEK 293 cells were grown in DMEM supplemented with 10% FBS, glutamine and antibiotics. For each construct, cells were plated on the day prior to transfection at 5 × 10^5 into 6-well plates and then transfected with 2 µL of Lipofectamine 2000 (Invitrogen) and 1 µg of the N or C-terminus constructs. As a transfection control we used a CD5-pEE14 construct that coded for the extracellular domain of CD5. Protein expression was analysed 2-3 days after transfection by Western blotting (WB) with a mouse monoclonal antibody anti-His6 (0.2 µg/ml, Roche).

Stable transfection of N-SSC5D, C-SSC5D and Spα

CHO-K1 cells were cultivated in DMEM supplemented with 10% FBS, 1 penicillin G (50 U/ml) and streptomycin (50 µg/ml), 4 mM of L-glutamine, 50x amino acids (AA) and nucleoside supplement and 1 mM sodium pyruvate. The day before transfection, cells were detached from the flasks using trypsin-EDTA (Sigma), divided into two 25 cm^3 flasks and plated at 4 × 10^6 cells per flask in the growth medium. One flask contained the cells to be transfected with each construct and a second flask was reserved for mock transfection. Cells were incubated overnight at 37 °C, 5% CO_2, to be 80% confluent on the following day. On the transfection day, the medium was removed and replaced with complete Glasgow Minimum Essential Medium (GMEM) (First Link) supplemented with 10% of dialyzed FCS (First Link), 50x AA and nucleoside supplement, antibiotics and deprived of L-glutamine. Cells were transfected with a mixture of 9 µL Lipofectamine 2000 (Invitrogen) in 500 µL Opti-MEM I (Gibco, Life Technologies) and 3 µg of each construct diluted in 500 µL of Opti-MEM I (final volume = 1 ml) according to the manufacturer's instructions. For the mock transfection, ddH_2O was used instead of DNA.

The DNA-Lipofectamine complex was added directly to the cells medium and mixed gently by rocking the flask back and forth. Cells were incubated at 37 °C in the CO_2 incubator overnight.

On the following day, cells were trypsinized and plated into 96-well flat-bottomed plates at 1 × 10^6 cells per plate in 100 µl volume per well. The plates were incubated overnight before the addition of the selection medium. Next, 100 µl of supplemented medium (no L-glutamine) containing the desired concentration of the selection reagent methionine sulphoximine (MSX) was added to each well. The final concentrations used

were 40 and 60 µM of MSX in 200 µl volume per well. Plates were incubated for one week at 37 °C in the CO_2 incubator. Afterwards, 100 µl of medium was removed from each well and replaced with 100 µl supplemented GMEM with the desired concentration of MSX. The plates were incubated at 37 °C for two additional weeks.

Selection of stable transfectants by dot-blot

The surviving colonies of CHO-K1 transfected cells were counted and compared with the mock transfection. Then,15 µl of the medium from stable pools were directly transferred to nitrocellulose membrane and tested for N-SSC5D, C-SSC5D or Spα recombinant expression by a dot-blot immunoassay using a mouse monoclonal antibody anti-His (0.2 µg/ ml, Roche) and a peroxidase-conjugated goat anti-mouse IgG antibody (1:20,000, Santa Cruz) and revealed using Amersham ECL detection reagent (GE Healthcare Life Sciences) and exposed to BioMax MR films (Kodak).

N-SSC5D-, C-SSC5D- and Spα-expressing clones were expanded in selective supplemented GMEM and were tested by western-blotting to confirm both protein expression and size. The high-expressing clones of each recombinant protein were frozen in liquid nitrogen. Additionally, one of each clone was grown to confluence in 10-tray cell factories (Nunc, Roskilde, Denmark) at 37 °C. Meanwhile, sodium butyrate was added to these factories to prevent cell overgrow. After 4 weeks, one litre of tissue culture supernatants (TCS) containing recombinant N-SSC5D and Spα were collected and stored at 4 ºC for protein purification.

Western blotting

Tissue cultures supernatants (TCS) of N-SSC5D, C-SSC5D and Spα were analysed by Sodium Dodecyl Sulfate Poly-Acrylamide (acrylamide 7.5%) Gel Electrophoresis (SDS-PAGE) under reducing conditions. To prepare each sample, 16 µl of TCS were mixed with 4 µl 5x SDS loading buffer with 10% mercaptoethanol and boiled at 95 ºC for 10 min. The gel was run for 1 h at 150 V with Tris/glycine/SDS running buffer (Bio-Rad Laboratories). In order to identify protein size, a protein standard (Bio-Rad Laboratories) was also loaded. Proteins were transferred to the nitrocellulose membrane (Hybond-C-extra) by electroblotting with ice-cold transfer buffer (1 h at 100 V). Membranes were blocked with TBS, 0.1% Tween 20 (TBS-T), containing 5% nonfat

dried milk for 1 h at RT. Then, the membrane was incubated with mouse monoclonal antibody anti-His6 (0.2 µg/ml, Roche) in TBS-T with 3% nonfat dried milk, for 1 h at RT. Membranes were washed thoroughly with TBS-T and then incubated with peroxidase-conjugated goat anti-mouse IgG in TBS-T with 3% nonfat dried milk (Santa Cruz, 1:20,000) for 1 h at RT. Immunoblot was developed using Amersham ECL detection reagent (GE Healthcare Life Sciences) and exposed to BioMax MR films (Kodak). To confirm byotinylation, nitrocellulose membranes were directly incubated with 1:5000 ExtrAvidin-Peroxidase® (Sigma) in TBS-T.

N-SSC5D immunoblotting using anti-SSC5D

For N-SSC5D immunoblotting, samples were run in SDS- PAGE for 1 h at 150 V with Tris/glycine/SDS running buffer (Bio- Rad Laboratories). Samples were transferred to the nitrocellulose membrane using the iBlotTM Gel Transfer Device (Invitrogen) following the manufacturer's instructions. Then, the membrane was blocked with TBS, 0.1% Tween 20 (TBS-T), containing 5% non-fat dried milk, for 1 h with shaking. N-SSC5D was subsequently detected with rabbit anti-SSC5D (Abgent, 1:5,000) primary antibody in TBS-T with 3% non-fat dried milk, for 1h at RT, followed by peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma, 1:30,000) for 1h at RT. The immunoblot was developed using ECL detection reagent (GE Healthcare Life Sciences), and the image was acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories).

Protein purification

N-SSC5D and Spα TCS were harvested and phenylmethylsulfonyl fluoride (PMSF) (Sigma) was added at 1 mM final concentration. Next, TCS were centrifuged at 10,000*g*, 20 min at 4 ºC, to remove cell debris and diluted 4 x in PBS. The histidine-tagged recombinant proteins N-SSC5D and Spα proteins were purified by immobilized metal affinity chromatography using Ni Sepharose™ High Performance HisTrap™ HP 1 ml column, (GE Life Sciences) using a BioLogic DuoFlow QuadTec10 System (Bio-Rad Laboratories). The imidazole gradient was run from 10 mM to 500 mM. The purest fractions containing N-SSC5D and Spα were collected and further purified by anionic chromatography (UNO Q column Bio-Rad) with a linear salt gradient (0–1.0 M NaCl) in PBS using AKTA Purifier (GE Life Sciences). Fractions containing the desired protein

were pooled and concentrated to 1 ml using Amicon® Ultra Centrifugal filters with 10 kDa cutoff (Milipore) (4000*g*, 20 min). Proteins concentrations were calculated using the Beer-Lambert equation ($A = \varepsilon * l * c$), where c is the molar concentration (M), l is the path length of the cuvette (units cm), ε is the molar extinction coefficient (units $M^{-1}cm^{-1}$) and A is the absorbance at a 280 nm wavelength. Absorbance was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and the molar extinction coefficients were predicted using the ExPASy Protparam.

Coomassie staining

N-SSC5D and Spα protein purity was analysed by SDS-PAGE (7.5 % acrylamide). To prepare each protein sample, 5-20 µl of the fractions obtained previously in the chromatography were mixed with 2x loading buffer containing SDS and 5% mercaptoethanol (Sigma). Next, the samples were boiled at 95 ºC for 10 min and loaded into the gel. The gel was run for 1 h at 150 V with Tris/glycine/SDS running buffer (Bio-Rad) to separate protein samples. In order to identify proteins by size, a protein standard (Bio-Rad) was also loaded. Once the proteins had separated, the gel was stained with Bio-Safe Coomassie Premixed Staining Solution (Bio-Rad) for 1 h and washed thoroughly with deionized water.

Proteins biotinylation and tetramers formation

Proteins were changed to biotinylation buffer (Tris-HCl 10 mM pH 8) using Amicon® Ultra Centrifugal filters with 10 kDa cutoff (Milipore) and biotinylated using the BirA enzyme (Avidity LLC) at 26 ºC overnight, following manufacturer's instructions. Next, buffer was exchanged to HBS buffer (25 mM HEPES, 140 mM NaCl, 1.42 mM Na_2HPO_4, 0.01% NaN_3 pH 7). To form the tetramers 200 µg/ml of proteins were incubated with 50 µg/ml (Alexa Fluor 647)-streptavidin (Invitrogen) in HBS buffer for 15 min at room temperature using a rotating wheel.

Flow cytometry

For the screening of cell surfaces ligands using the tetrameric forms of proteins, 1×10^6 cells per sample were collected, centrifuged at 450*g* for 10 min and washed twice with

FACS buffer (PBS containing 0.2% BSA and 0.05% NaN_3). Cells were then plated in 96-well plates round bottom, centrifuged at 450*g*, 5 min at 4ºC to remove media and incubated with 30 µl of tetramers for 45 min in the dark on ice. Next, cells were washed twice with FACS buffer (450*g*, 5 min, 4ºC). Finally, cells were resuspended in PBS, 0.05%, NaN_3, 1% paraformaldehyde. As controls we used unstained cells and cells incubated with (Alexa Fluor 647)-streptavidin (no protein added). Flow cytometric analysis was performed using a FACS Calibur I flow cytometer (BD Immunocytometry Systems).

1.3. Results

The N-SSC5D (encoded by exons 1-12) and C-SSC5D (encoded by exons 13-14) polypeptides were produced individually due the structural and potentially functional differences between both domains (Fig. 1.1). In addition, Spα was also analysed because it resembles N-SSC5D. Spα is composed exclusively of SRCR domains and like SSC5D is a secreted protein abundant in serum. Therefore, we sought to know whether these proteins had a similar binding profile, which could indicate a common ligand for SRCR domains in soluble proteins.

Production of the N- and C-terminal domains of SSC5D

Figure 1.1. Schematic representation of the N-terminal (N-SSC5D) and C-terminal (C-SSC5D) domains of SSC5D. The molecular weight (MW) of the N-SSC5D and C-SSC5D domains were predicted based on their amino acids composition using the bioinformatics resource portal ExPASy.org (http://web.expasy.org/compute_pi/). The predicted MW of the N-SSC5D and C-SSC5D domains are of 100 and 65 kilodaltons (kDa), respectively.

The cDNA fragments encoding N-SSC5D and C-SSC5D were cloned into a modified version of the commercial mammalian expression vector pEE14-BirA (Lonza Biologics) (Fig. 1.2). The selected vector permits the permanent integration of foreign genes into chromosomes of mammalian cell lines, which are then replicated. Moreover, eukaryotic

systems are capable of expressing and secreting post-translational modified proteins, a fundamental requisite especially regarding the C-terminal domain of SSC5D that is predicted to be extensively *O*-glycosylated. pEE14-BirA vector contains an N-terminal CMV promotor and hemagglutinin (HA) affinity tag, followed by C-terminal biotin ligase (BirA) recognition site and hexahistidine (6x His) affinity tag.

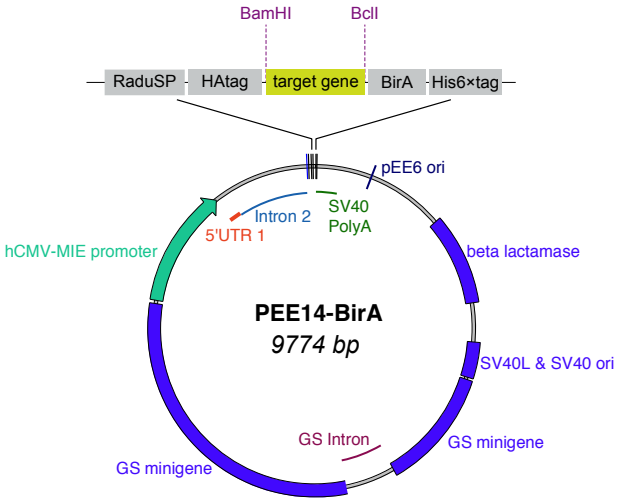

Figure 1.2. Cloning strategy of the constructs encoding the N-SSC5D, C-SSC5D or Spα for stable expression of recombinant proteins. A modified version of pEE14 plasmid was used to clone N-SSC5D, C-SSC5D or Spα constructs for soluble recombinant protein expression in HEH293 and CHO-K1 cells. The final product is translated as a single string of amino acids with the following order, a signal peptide (RaduSP), a HA-tag, the N-SSC5D or C-SSC5D or Spα sequences, a BirA (BirA) recognition sequence and a 6x His tag (His6xtag).

N- and C-SSC5D plasmids were transiently transfected into HEK 293 cells using a cationic lipid-based transfection reagent (Lipofectamine). This small-scale analysis intended to confirm the expression and molecular weight of N-SSC5D and C-SSC5D. As proteins are expected to be secreted, tissue culture supernatant (TCS) was harvested 3 days after transfection and WB analysis using an antibody anti-His was performed. As a transfection control, we used a similar construct encoding the extracellular domain of CD5. Nonetheless, we could not detect the expression of the N-SSC5D or C-SSC5D using this fast strategy. However, we observed CD5 expression suggesting that the transfection protocol was efficient (Fig. 1.3). The predicted MW of CD5 corresponds to approximately 46 kDa.

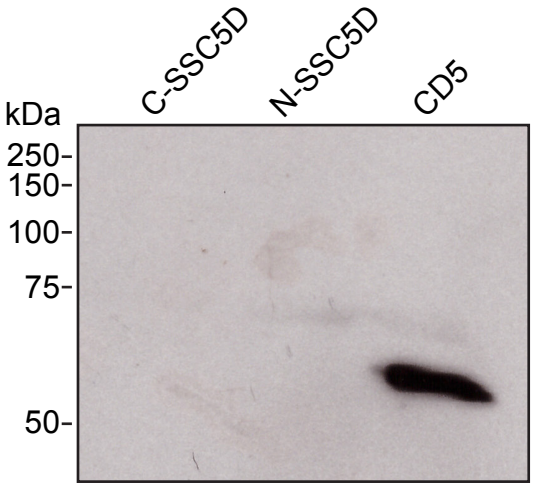

Figure 1.3. SDS-PAGE (7.5 %) immunoblotting of the TCS of C-SSC5D, N-SSC5D and CD5 using an anti-His antibody followed by a peroxidase-conjugated goat anti-mouse. The supernatant of CD5 presents a single band at approximately 50 kDa.

Since we were not able to induce strong protein expression using transient transfections, we attempted a stable transfection system using CHO-K1 cells. For the production of large amounts of proteins, a long-term gene expression is usually preferred. To develop a stable transfection system, it is necessary to use a selection method, for example by co-expressing a specific gene that allows the selection of the gene that produces the desired recombinant protein. pEE14 codes for glutamine synthetase (GS), an enzyme that in the presence of the substrates glutamate and ammonia is able to produce glutamine, an essential amino acid required for cell growth and survival. The activity of the GS enzyme is selectively inhibited by methionine sulphoximine (MSX), so only the cells that incorporate the vector and express GS at adequate levels in a glutamine-free media will survive in long-term cultures, allowing for the selection and expansion of the cells expressing the recombinant protein (Cockett et al., 1990, Blochberger 1997). After stable transfection, cells were cultured into 96-well plates and selected with MSX (see Methods). To confirm protein expression by MSX resistant clones, we performed a dot-blot (see Methods). However, once more we could not observe protein expression. One hypothesis was that the proteins could be retained within cells; therefore, we decided to expand clones in T25 cm^3 culture flasks. At this stage, we collected both TCS and lysate from the clones. The cell lysates were negative for both N- and C-SSC5D expression; however, the TCS of one N-SSC5D clone - E6 - presented a very faint band with a MW above 150 kDa that could correspond to the N-terminal domain (Fig. 1.4)

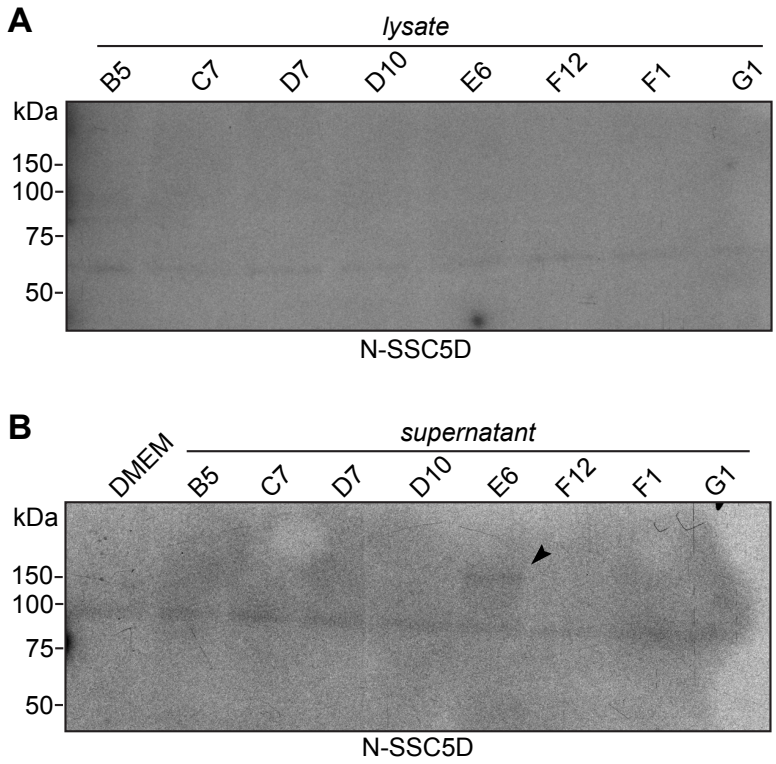

Figure 1.4. SDS-PAGE (7.5%) immunoblotting of the lysates (A) and TCS (B) of eight N-SSC5D clones using an anti-His antibody followed by a peroxidase-conjugated goat anti-mouse. The supernatant of E6 presents a single faint band with a MW above 150 kDa.

Purification of N-SSC5D

To confirm whether the faint band corresponded to N-SSC5D, the E6 clone was grown to confluence in a T75 cm^3 culture flask. In order to concentrate the protein, the TCS was run over a nickel affinity column and proteins were eluted with imidazole (Fig. 1.5). To detect the presence of N-SSC5D in the eluted fractions we run a SDS-PAGE immunoblotting using an anti-His antibody.

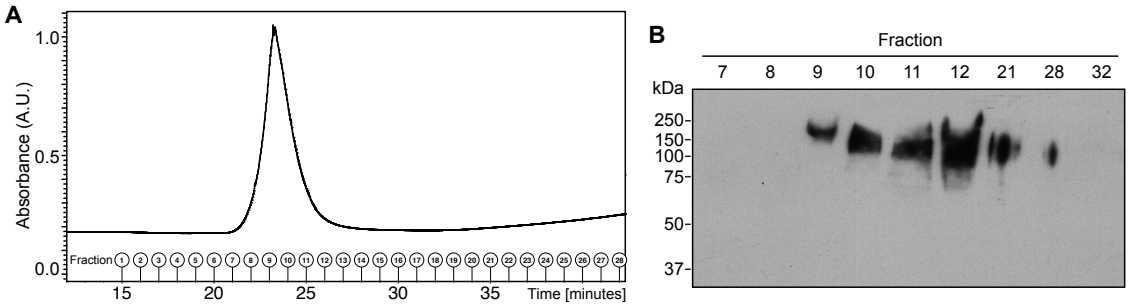

Figure 1.5. The TCS of the E6 clone expressing N-SSC5D was run over a nickel affinity column. **A.** N-SSC5D protein was eluted with 50 mM imidazole in PBS. **B.** SDS-PAGE (7.5 %) immunoblotting of 7-12, 21, 28 and 32 fractions using an anti-His antibody followed by a peroxidase-conjugated goat anti-mouse.

N-SSC5D expressing cells were then cultivated in cell factories for large-scale protein production, then secreted N-SSC5D was purified from TCS by nickel affinity chromatography using increasing concentrations of imidazole. Fractions from 20 mM – 500 mM were run in SDS-PAGE and stained with Coomassie blue. However, N-SSC5D began to be eluted with low imidazole concentrations (20-40 mM), which resulted in a higher number of contaminants in the fractions containing N-SSC5D. To remove all contaminants, the purest fractions were collected to perform an ionic exchange chromatography (Fig. 1.6). The fractions obtained with this technique yielded pure N-SSC5D, which was concentrated and quantified, the final yield being of approximately 0.3 mg per one liter of culture medium.

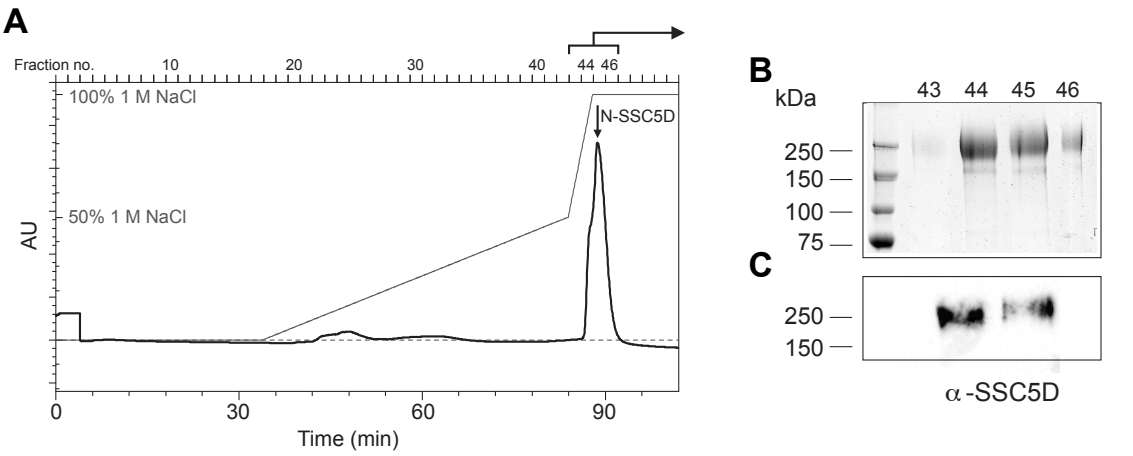

Figure 1.6. Anion exchange chromatography purification of N-SSC5D using an Uno-Q column. **A.** The purest fractions resulting from the metal-chromatography were collected and subsequently loaded onto an Uno-Q column. The slope is the conductivity of the eluate, representing the salt gradient (0 - 1 M NaCl in PBS). **B.** SDS-PAGE (7.5 %, under reducing conditions) of 43-46 fractions. The fractions were stained with Coomassie blue to confirm protein purity. **C.** SDS-PAGE immunoblotting using an anti-SSC5D antibody followed by peroxidase-conjugated goat anti-rabbit IgG antibody.

Production of C-SSC5D

Regarding the mucin-like domain, WB analysis of single clones TCS showed a pattern of several bands with different sizes (predicted MW ~ 65 kDa). This pattern could be explained by C-SSC5D *O*-linked oligosaccharides, protein aggregation or degradation (Fig. 1.7). Protein purification was not feasible, so we abandoned the C-SSC5D screen analysis and production.

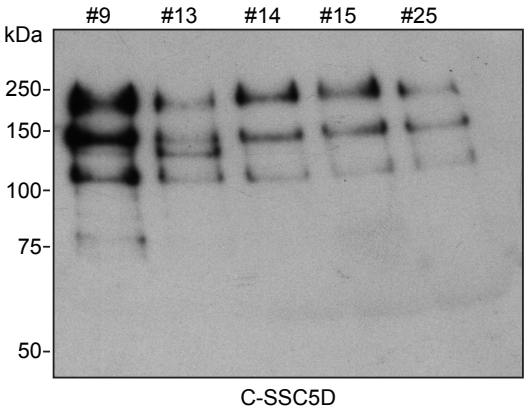

Figure 1.7. Analysis of C-SSC5D protein expression. TCS from five MSX resistant C-SSC5D clones were run in SDS-PAGE, 7.5 %, under reducing conditions. Detection of C-SSC5D was performed using an anti-His antibody followed by a peroxidase-conjugated goat anti-mouse.

Production and purification of Spα

CHO-K1 cells were transfected with the Spα plasmid and cultured on 96-well plates. After MSX selection, 15 μl of TCS from the surviving clones were tested for Spα expression (dot blot) (Fig. 1.8A). Seven clones were selected and grown to 6-well plates (Fig. 1.8B). Next, to confirm protein size, TCS samples were loaded on an SDS-PAGE gel to perform WB using an anti-His antibody.

We observed that all clones presented a band of 50 kDa with a higher size than the expected (predicted MW ~ 38 kDa) probably derived from complex glycosylation.

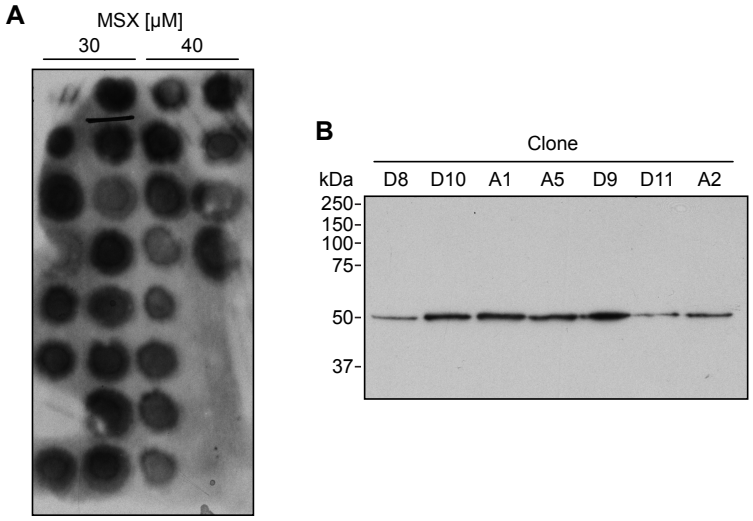

Figure 1.8. Dot blot analysis of Spα protein expression and immunoblotting of Spα. **A.** Nitrocellulose membrane was blotted with the TCS of MSX resistant Spα clones. **B.** TCS from seven different clones were run in SDS-PAGE, 7.5%, under reducing conditions. Detection of Spα was performed using an anti-His antibody followed by a peroxidase-conjugated goat anti-mouse.

The A1 clone was selected and grown until the cell factory phase. Next, TCS was harvested and pure protein was obtained by following the 2-step purification protocol described for N-SSC5D (Fig. 1.9). The final yield of Spα was approximately 1 mg per one liter of culture medium.

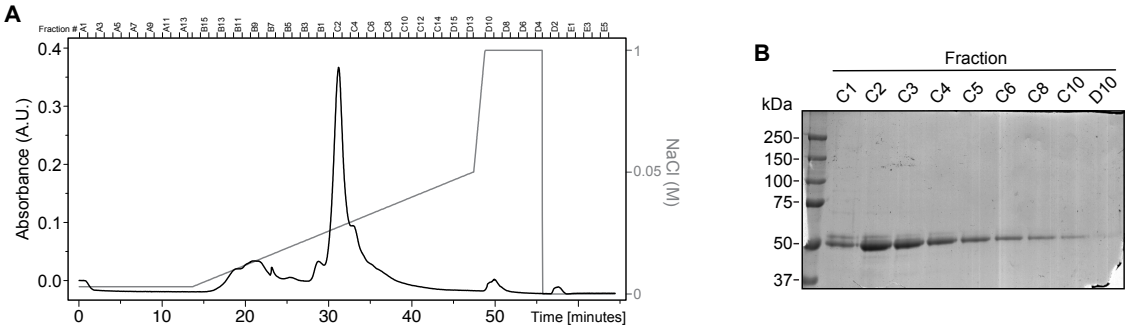

Figure 1.9. Anion exchange chromatography purification of Spα using an Uno-Q column. **A.** The purest fractions resulting from the metal-chromatography were collected and subsequently loaded onto an Uno-Q column. The slope is the conductivity of the eluate, representing the salt gradient (0 - 1 M NaCl in PBS). **B.** SDS-PAGE (7.5%, under reducing conditions) of C-1 – D10 fractions. The fractions were stained with Coomassie blue to confirm protein purity.

Screening of cell lines with tetramers of N-SSC5D and Spα

To increase the sensitivity of potential SSC5D and Spα interactions with cell surface ligands, multimeric forms of both proteins were assembled. N-SSC5D and Spα recombinant proteins have a biotin ligase (BirA) recognition site that was then biotinylated using a biotin ligase enzyme. Next, biotinylated N-SSC5D and Spα were incubated with streptavidin, a tetrameric protein composed of four identical subunits, where each subunit can bind with high affinity one biotin molecule independently. This ligation allowed the formation of N-SSC5D and Spα tetramers. To confirm protein biotinylation we performed an immunoblotting using the biotin binding protein ExtrAvidin-Peroxidase ® (Fig. 1.10).

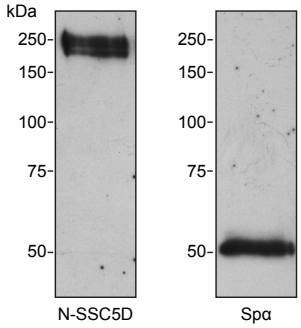

Figure 1.10. Analysis of N-SSC5D and Spα biotinylation. Following biotinylation, proteins were run in a SDS-PAGE, 7.5%, under reducing conditions. After transferring N-SSC5D and Spα from the gel to the nitrocellulose membrane, proteins were directly incubated with ExtrAvidin-Peroxidase ®. The W.B. show the bands corresponding to the biotinylated N-SSC5D (left) and Spα (right).

Mucosal surfaces of the body are especially vulnerable to infection. They are thin and permeable barriers to the interior of the body and are in contact with the exterior because of their physiological activities such as food absorption (the gut) and reproduction (uterus). Accordingly, cell surface ligands derived from colon (Caco-2, HCT-116) and uterus (HeLa) appeared as prospective candidates for SSC5D and Spα binding. Moreover, taking into account the high expression of SSC5D in placenta and of its mouse homologue in the urogenital tract, cell lines derived from these tissues (JEG-3 and HEK293) were also screened. In addition, the tetramers of SSC5D and Spα were also incubated with cells of immune origin (Jurkat E6.1, K562, THP-1) (Table 1.1).

Table 1.1. Cell lines used in the flow cytometry screening.

Caco-2	Human epithelial colorectal adenocarcinoma
HCT-116	Human colon carcinoma
HEK 293	Human embryonic kidney
HeLa	Human epithelial cervix carcinoma
JEG-3	Human placental choriocarcinoma
Jurkat E6.1	Human acute T cell leukemia
K562	Human erythromyeloblastoid leukemia
THP-1	Human monocytic leukemia

From the results obtained we were not able to see any detectable binding of N-SSC5D to the cell lines selected (below the detection level of this assay) (Fig 1.11) Nonetheless, we cannot exclude the existence of a cell surface receptor for N-SSC5D in other cell types; it may therefore be relevant to perform a wider screen analysis. Interestingly, tetrameric Spα binds to JEG-3 and Caco-2 cells, which suggests that there is a putative ligand for Spα in placenta and intestine cells. On the contrary, we were expecting an interaction between Spα and K562; however the Spα-K562 interaction is practically negligible. Possible reasons for this result are addressed in the discussion section.

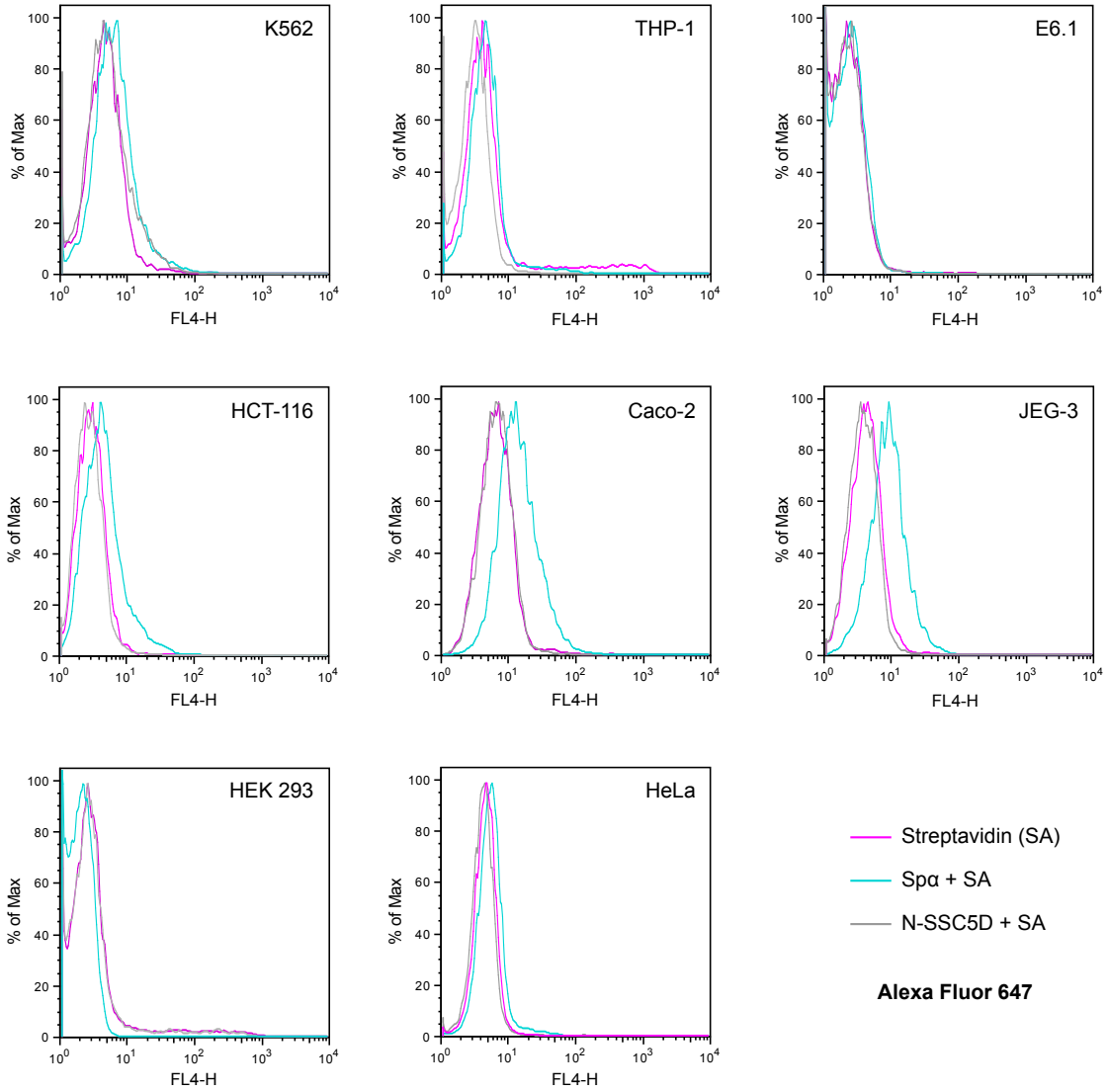

Figure 1.11. Flow cytometry screening of different cell lines with N-SSC5D and Spα tetramers**.** Cells were incubated with N-SSC5D and Spα tetramers. N-SSC5D (grey), Spα (blue) and uncoupled (Alexa Fluor 647)-streptavidin (purple).

1.4. Discussion

Protein interactions control the outcome of most cellular processes, including immunity and disease aetiology. Therefore, identifying and characterizing protein recognition events is essential to understand the biological function of immune receptors. Despite most protein-protein interactions being highly specific, many proteins form transient complexes that are difficult to identify (Shoemaker and Panchenko, 2007). Hence, to identify potential interactions between SSC5D and cell surface ligands, we used a

systematic approach that consisted on the production of recombinant proteins assembled in a multimeric form to increase the assay sensitivity.

However, using eukaryotic expression systems to produce functional recombinant proteins in satisfactory quantities can be time-consuming and difficult. In our experiments we used two cell lines, CHO-K1 and HEK293, with the capacity for carrying out posttranslational modifications, a feature essential for the analysis of rSSC5D that is predicted to be heavily glycosylated (Gonçalves et al., 2009). We started by using transient transfection (HEK293), because it requires less time to express proteins (Brondyk, 2009). However, we could not observe the expression of SSC5D, even though we assume that the protocol worked, as we could detect sCD5 expression. Although a stable transfection system can take 2–3 months to generate a single cell line expressing proteins (Brondyk, 2009), we attempted to obtain N- and C-SSC5D by integrating these genes in the CHO-K1 cell genome. This strategy was successful, albeit the yield of N-SSC5D obtained after protein purification was very low (0.3 mg), when compared with Spα (1 mg) and with sCD5 (18 mg) (which was previously expressed using the same expression system by Santos (2012)). We did several attempts to increase protein recovering, including adapting cells to serum free media to minimize the loss of protein during purification steps, and tried other commercial transfection reagents; still, the yield of N-SSC5D was significantly low. One possible explanation is that the insertion of the N-SSC5D gene fragment in the CHO-K1 genome was not in a high-quality locus.

Regarding C-SSC5D, we observed several clones expressing the protein; however, we were not able to purify C-SSC5D due of its intrinsic nature. Its high glycosylation pattern that confers SSC5D a mucin-like structure formed massive aggregates that would hardly correspond to the native C-terminal domain of SSC5D. Glycosylation affects the outcome of a protein by changing its biophysical properties, such as charge, folding, solubility or sensitivity to proteases (Varki, 1993).

The screening assay showed that none of the cell lines analysed bound N-SSC5D. Nonetheless, at this stage it is not possible to draw definitive conclusions about SSC5D interactions with cell surfaces. Firstly, we cannot exclude that cells from other origins would give a different result. Secondly, as we could not fully address C-SSC5D interactions, one cannot discard cell interactions through this domain. Thirdly, N-SSC5D is a recombinant protein, so it may present a conformation that hampers the binding of potential ligands. Moreover, protein–protein complexes can have high dissociation constants, therefore the binding affinity of recombinant proteins may not

be enough to perceive an interaction. Nevertheless, this was the first genuine attempt to find a cell surface ligand for SSC5D using a systematic protocol. This study was intended to discover cell surface ligands, so in the eventually of SSC5D not binding to cells, there is still a myriad of soluble proteins and molecules that can interact with SSC5D and contribute to its biological function.

Interestingly, mouse SSC5D was shown to bind galectin-3 (Miró-Julià et al., 2014), a 30-kDa protein that is ubiquitously expressed and is present in the cytoplasm, nucleus, extracellular space, and cell surface (Dumic et al., 2006). Galectin-3 binds β-galactoside sugars and is involved in diverse cellular processes including the modulation of signal transduction events on the cell surface. One of its ligands is the Mac-2-binding protein (M2BP) (Inohara et al., 1996), a heavily *N*-glycosylated secreted protein that is a class A SRCR-SF protein. This glycoprotein is also found in sera and has a predicted molecular weight of 97 kDa; however, it has a high tendency to associate and therefore oligomers can exhibit average molecular masses between 1000 and 1500 kDa (Müller et al., 1999). In a pioneer study using a melanoma cell line, affinity purified M2BP bound cell surface galectin-3 in a specific carbohydrate-dependent manner (Inohara et al., 1996). One other study showed that MUC2, an intestinal mucin, is a major ligand for galectin-3, whose interaction is much associated with colon cancer (Dudas et al., 2002). Therefore, we may suspect that galectin-3 could bind, as its mouse counterpart, native human SSC5D (probably through the mucin-like domain). However, as it happens with the CD6-CD166 interaction, we cannot discard the existence of a specific cell ligand for SSC5D, which could interact through SRCR domains.

On another note, we showed for the first time the binding of Spα to nonimmune epithelial cells, Caco-2 and JEG-3, a colon and a placenta derived cell lines, respectively. Despite that two studies have observed that Spα bound to the myeloid cell line K562, in our hands this interaction was practically negligible (Gebe et al., 1997; Sarrias et al., 2004). Curiously, there is a noteworthy difference between our study and the previous ones. In the studies where Spα interacts with K562 cells, the interaction happens in the presence of calcium, which may help to modulate specific ligand interactions.

Current views suggest that the multifunctional and widely expressed CD36 molecule is a cell receptor for Spα (Sanjurjo et al., 2015). However, CD36 recognizes a multitude of ligands, ranging from bacterial cell-wall components to endogenous lipoproteins, and therefore the biological significance of the interaction between Spα and CD36 is

unclear (Sanjurjo et al., 2015). Nonetheless, there are cells that do not express CD36 but are still susceptible to Spα actions (Maehara et al., 2014; Sanjurjo et al., 2015). Therefore, other cell surface receptors, namely from the complement system, have been proposed as ligands for Spα. Moreover, a study conducted to localize CD36 in the human gastrointestinal tract showed that CD36 transcripts were absent in Caco-2 cells (Lobo et al., 2001). Altogether, these studies suggest that Spα may indeed have alternative cell surface ligands.

1.5. References

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Chapter 2

The scavenger receptor SSC5D physically interacts with bacteria through the SRCR-containing N-terminal domain

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2.1. Introduction

The early detection of pathogens by the immune system is mediated by germline-encoded pattern-recognition receptors (PRR), which recognize a variety of conserved pathogen-associated molecular patterns (PAMP) that range from bacterial and fungal cell-wall components to viral nucleic acids (Janeway Jr, 1989; Iwasaki and Medzhitov, 2015). Microbes have evolved diverse traits that enhance their ability to spread and foster disease in a susceptible host (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2001). This capacity to cause infection is provided by diverse virulence factors that include surface membrane proteins, responsible for adhesion, colonization and invasion *e.g.*, pili and adhesins, polysaccharide capsules with anti-phagocytic properties, and secretory proteins, like toxins and enzymes (Finlay and Falkow, 1997; Wu et al., 2008). Such diversity of structures is specifically recognized by membrane-bound, cytosolic or secreted molecules belonging to several classes of PRR, including Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, and C-type lectin receptors (CLR), among others (Areschoug and Gordon, 2009; Blander and Sander, 2012; Iwasaki and Medzhitov, 2015).

Accumulating evidence suggests that members of the SRCR superfamily represent an important part of the innate immune defence by acting as PRR, exploiting diverse mechanisms that may help to ward off infection (Areschoug and Gordon, 2009; Martínez et al., 2011; Blander and Sander, 2012; Kneidl et al., 2012). Accordingly, four of the nine human receptors belonging to the group B of the SRCR superfamily have been shown to bind bacteria or bacterial components. Bacterial interactions were reported for the T cell receptor CD6, the monocyte/macrophage receptor CD163, DMBT1, which has a broad expression profile, and Spα, a soluble glycoprotein expressed by macrophages in the lymphoid tissues and highly present in the serum [detection levels of microgram per millilitre (Sarrias et al., 2004)] (Prakobphol et al., 2000; Sarrias et al., 2005; Sarrias et al., 2007; Fabriek et al., 2009; Madsen et al., 2010; Martínez-Florensa et al., 2013; Polley et al., 2015). Conversely, the T cell receptor CD5 lacked the ability to bind bacteria, but was shown to interact with conserved fungal components and to aggregate fungal cells (Vera et al., 2009).

After bacterial challenges, the soluble mouse homologue of Spα (AIM) is immediately released from macrophages to control bacteria spreading and, similar to Spα, reduces

inflammatory cytokine secretion by PRR-expressing innate cells (Sarrias et al., 2005; Martinez et al., 2014). *In vivo* studies of PAMP-induced septic shock have shown that the levels of the AIM increase rapidly upon injection of LPS or Zymosan, further suggesting that this SRCR protein can act as a circulating PRR (Martinez et al., 2014). SSC5D is a less-explored soluble member of the SRCR family that shares many features with Spα. SSC5D is expressed in macrophages, T cells, and several epithelial cells, especially from placenta, spleen, and colon (Gonçalves et al., 2009). It is also highly abundant in the serum with increasing levels in inflammatory conditions (Balakrishnan et al., 2014). The mouse homologue of SSC5D [S5D-SRCRB] is also upregulated upon infection and seems capable to bind bacteria (Miró-Julià et al., 2014); however, for the human counterpart this capacity has not been reported. A major difference between SSC5D and Spα relates not only to the number of SRCR domains (5 and 3, respectively) but also to the existence of a large mucin-like sequence located at the C-terminus of SSC5D. In the human molecule, this domain represents about 40% of the amino acid content of the whole protein and it is expected that, similar to other *O*-glycosylated mucin-like proteins, it may bind and modulate pathogen behaviour (Linden et al., 2008; van Kooyk and Rabinovich, 2008; McGuckin et al., 2011).

Since the late 1990's, a leading tool for the study of biomolecular interactions both in life science and pharmaceutical research are biosensors (Piliarik et al., 2009). Label-free biosensors have revolutionized the qualitative and quantitative analysis of biomolecular interactions (*e.g.*, protein–protein or protein–nucleic acids interactions) and are increasingly being adopted in medical diagnostics, environmental monitoring, or food safety and security (Homola, 2008; Mariani and Minunni, 2014). Briefly, biosensors consist of analytical devices that perceive biomolecular interactions and transduce these interactions into digital signals that can be interpreted by the users.

Today, one of the leading devices used in biomedical research are optical sensors based on surface plasmon resonance (SPR), a phenomenon arising from the interaction of light with free electrons, the surface plasmons (SP), at a metal-dielectric interface (Homola, 2008). Surface plasmons are a collective oscillation of free electrons inherently coupled to an electromagnetic wave, propagating at the interface of a thin metal layer (~50 nm), usually gold, and a dielectric medium (*e.g.*, aqueous solution) (Kretschmann and Raether, 1968; Homola, 2008). The charge density wave, named surface plasmon wave (SPW), is maximum at the interface and decays evanescently into both media. When a light wave hits a thin metallic surface, photons

of the incident light can, at specific conditions, resonantly transfer energy to the surface plasmons (Wood, 1902; Homola et al., 1999). This transfer of the energy appears as a narrow dip in in the spectrum of the reflected light. Owing that the propagation constant of the electromagnetic waves are highly sensitive to changes in the refractive index in the vicinity of the metal surface, the binding of biomolecules (analytes) to surface immobilized receptors (ligands) may induce changes in the propagation constant of the surface plasmon waves (Homola et al., 1999). The change in the propagation constant is determined by measuring changes in one of the characteristics of the light wave interacting with the surface plasmons, such as the wavelength, the incident angle or the intensity of the reflected light (Homola, 2008). The principle of SPR biosensing is therefore measuring changes in the refractive index at the sensor surface, caused by analyte binding to the chip surface-immobilized receptor. The most common interacting molecules include antibodies, peptides, DNA or RNA aptamers, and polymers, which are chosen depending on the target (analyte) to be bound (Homola, 2008).

There are several configurations of SPR apparatus that are capable of generating and measuring surface plasmon resonance. Figure 2.1 illustrates a multi-channel SPR sensor based on the Kretschmann configuration of the attenuated total reflection (ATR) method (Kretschmann and Raether, 1968) and wavelength modulation (Homola, 1995). In the Kretschmann geometry, a thin a metal layer, typically gold with 50 nm of thickness, is placed on the top of a highly refractive glass prism surface (Kretschmann and Raether, 1968). When parallel polarised light totally reflects internally at the base of the prism generates an evanescent wave that passes through the metal layer and excites the surface plasmon waves at the interface of the metal (Homola et al., 1999; Homola, 2008). Interactions between the receptors immobilized on the chip surface and the target biomolecules in aqueous solutions change the refractive index resulting in a shift in the resonant wavelength of the incident light (Fig. 2.1B) The shift in the resonant wavelength is measured in real time and is proportional to the refractive index change at the sensor surface and thus to the surface concentration of captured biomolecules (Fig. 2.1C) (Homola et al., 1999; Homola, 2008).

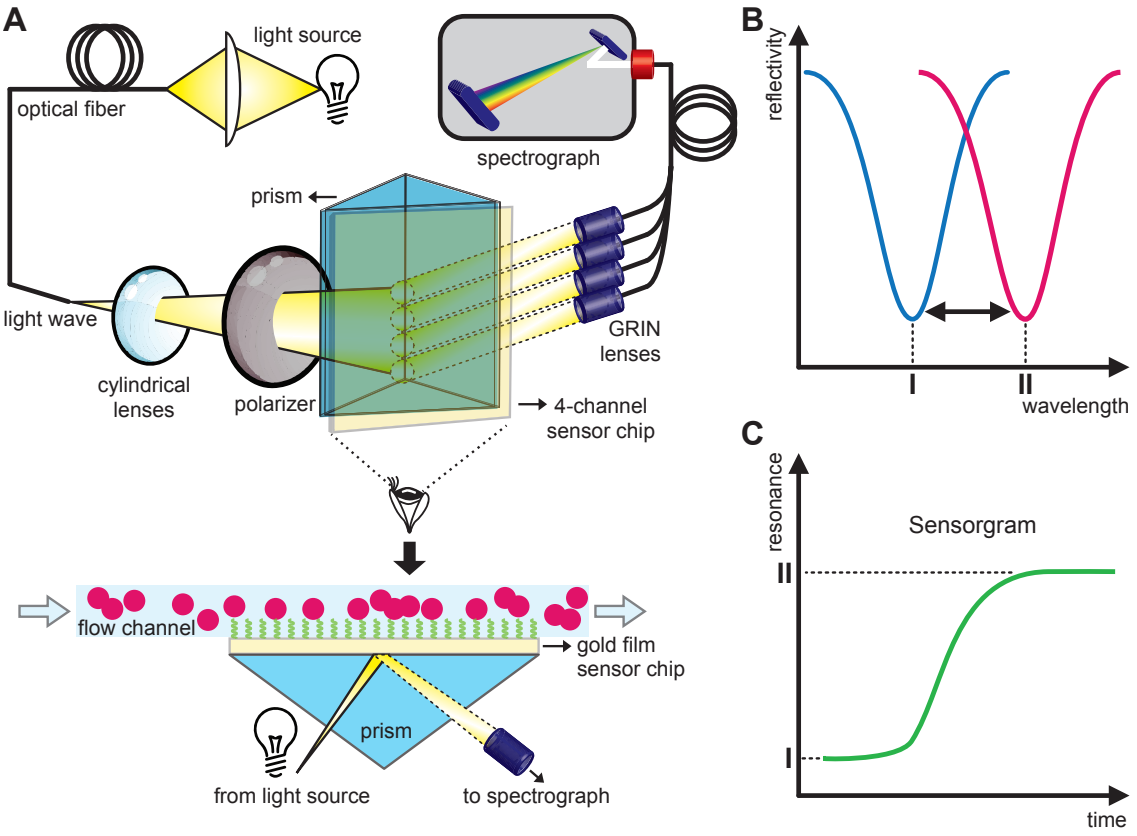

Figure 2.1. A. Scheme of a four-channel prism-based SPR sensor with wavelength modulation (adapted from Taylor 2006). A polychromatic light wave is directed through the prism at a fixed angle to the gold-coated surface where it excites SPs at the metal–dielectric interface. The strength of coupling between the incident wave and SPs is observed at multiple wavelengths and the wavelength yielding the strongest coupling is measured and used as a sensor output (Homola 2006). **B.** The SPR spectrum is shown in terms of reflectivity as a function of wavelength. The interaction between molecules immobilized on the chip surface and their targets increases the refractive index of the incident light resulting in a shift in the resonant wavelength. **C.** The increase in the refractive index is measured in real time and the result plotted as response or resonance units (RU) versus time (a sensorgram).

This highly sensitive detection technology that allows label-free and real-time studies of molecular binding processes is also employed in the detection of bacteria and other microbial pathogens (Homola et al., 2002; Bergwerff and Van Knapen, 2006; Taylor et al., 2006; Ahmed et al., 2014). SPR-based sensors for bacteria detection usually rely on the use of high-affinity antibodies recognizing particular components of bacterial surfaces (Dudak and Boyacı, 2009). Despite the considerably weaker binding affinities for common receptor–ligand pairs when compared with antibody–antigen interactions, we hypothesized that an analogous strategy could be set up to scrutinize the interaction of secreted SRCR proteins with bacteria if these interactions were strong enough, reflecting a potential PRR nature of SRCR proteins.

Given that we did not find cellular targets for SSC5D binding (Chapter 1), we conjectured that, given the potential of a PRR-like function assigned to the SRCR family, SSC5D might interact with bacteria. In this work, we have developed a multi-channel SPR-based protocol to test 1) the ability of SPR biosensor technology to monitor the interaction of secreted SRCR proteins with whole cell bacteria of different types and 2) the bacteria-binding capacity of the N-terminal moiety of SSC5D (excluding the mucin-like sequence likely to bind bacteria *per se* (Linden et al., 2008; van Kooyk and Rabinovich, 2008; McGuckin et al., 2011) and compared with the equivalent domains of other SRCR-family proteins, namely Spα and the extracellular domains of CD5 and CD6.

2.2. Materials and Methods

Recombinant protein production and purification

N-SSC5D and Spα production and purification is described in chapter 1. The soluble extracellular domain of CD6 (sCD6) and CD5 (sCD5) were produced as previously described (Oliveira et al., 2012; Santos, 2012). The protein purity of sCD6 and sCD5 is demonstrated in Figure 2.4. Protein purity was analysed by SDS-PAGE. Samples of the fractions obtained by chromatography were run for 1 h at 150 V, and the gel was stained with Coomassie Premixed Staining Solution (Bio-Rad Laboratories) for visualization of the protein product.

Bacteria strains

Listeria monocytogenes EGD-e was grown in brain heart infusion (BHI) medium (BD-Difco) at 37 °C to an optical density of 0.6 at 600 nm (OD_{600}; exponential phase), and *Escherichia coli* strains [BL21(DE3), IHE3034, RS218] were grown in Luria broth medium at 37 °C to an OD_{600} of 0.45.

2.2.1. Conventional bacteria–protein binding assays

Recombinant proteins Spα, N-SSC5D, sCD6, and sCD5 (5 µg per assay) were incubated for 1 h at 4 °C with the indicated cell suspensions of live bacteria (1×10^8 cells) in binding buffer (TBS, 1% BSA, 5 mM $CaCl_2$). Suspensions were centrifuged at 4,000*g* for 5 min at 4 °C. Cell pellets were washed thoroughly, then resuspended in 40 µl Laemmli's sample buffer, and denatured by heating at 95 °C for 10 min. Next, 20 µl of this lysate and pure recombinant proteins (25 or 100 ng) were separated in 6% SDS-PAGE. The gel was run for 1 h at 150 V with Tris/glycine/SDS running buffer (Bio-Rad Laboratories). After the SDS-PAGE, proteins were transferred to the nitrocellulose membrane using the iBlotTM Gel Transfer Device (Invitrogen) following the manufacturer's instructions. Then, the membrane was blocked with TBS-T containing 5% non-fat dried milk, for 1 h. Cell-bound protein was subsequently detected using mouse IgG1 anti-HA (clone 16B12) from Covance (0.1 µg/ml) in TBS-T with 3% non-fat dried milk, for 1 h at RT, followed by goat anti-mouse HRP-conjugated (Santa Cruz Biotechnology) (0.02 µg/ml) in the same conditions. The immunoblot was developed using ECL detection reagent (GE Healthcare Life Sciences), and the image was acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories).

2.2.2. SPR-based detection of whole bacterial cell interaction with SRCR proteins

Reagents

11-Mercapto-tetra(ethyleneglycol)undecanol ($HSC_{11}(EG)_4OH$) and 16-mercapto-hexa(ethyleneglycol) hexadecanoid acid ($HSC_{11}(EG)_6OCH_2COOH$) were purchased from Prochimia (Gdansk, Poland). Ethanolamine hydrochloride (EA), 3-(ethyliminomethylideneamino)-N,N-dimethyl-propan-1-amine (EDC), and 1-hydroxypyrrolidine-2,5-dione (NHS), all included in the Amine Coupling Kit, were purchased from Biacore (Uppsala, Sweden). All other chemicals were of analytical grade. Buffers used were SA_{10} (10 mM sodium acetate, pH 4.0/5.0), PBS (10 mM phosphate, 2.9 mM KCl, 137 mM NaCl, pH 7.4), PBNa (PBS** : 10 mM phosphate, 2.9 mM KCl, 750 mM NaCl, pH 7.4), and Tris (10 mM Tris-HCl, pH 7.4). All buffers were prepared using double glass-distilled and deionized water on Milli-Q50 (Millipore, Prague, Czech Republic).

SPR sensor

We used a laboratory four-channel SPR platform based on the wavelength spectroscopy of surface plasmons (Plasmon IV) (Pimková et al., 2012) developed at the Institute of Photonics and Electronics, Czech Republic. In this SPR biosensor, the sensor response is expressed as a shift in the wavelength of SPR resonance and is directly proportional to the mass of biomolecules attached to the surface of the sensor. Using the calibration procedure described in (Homola, 2006), the surface density of both the immobilized receptors and the subsequently attached molecules can be determined. For an SPR resonance of around 750 nm, the shift of 1 nm in the SPR wavelength represents a change in the protein surface coverage of 17 ng/cm^2 (Homola, 2006). All the experiments were performed at 25 °C.

Immobilization of proteins on SPR chip

The sensor chip was functionalized with a mixed self-assembled monolayer (SAM) by incubating the cleaned gold chip in degassed absolute ethanol with a mixture (7:3) of $HSC_{11}(EG)_4OH$ and $HSC_{11}(EG)_6OCH_2COOH$ alkanethiols at a final concentration of 200 μM. The $HSC_{11}(EG)_6OCH_2COOH$ alkanethiols terminated with a carboxylic head group were used to anchor a receptor by amino coupling, while $HSC_{11}(EG)_4OH$ alkanethiols terminated with hydroxylic group were used to form a stable non-fouling background. For that purpose, the sensor chip was immersed in a mixed thiol solution at a temperature of 40 °C for 10 min and then stored overnight in the dark at RT. After the formation of the mixed SAM, the chip was removed from the solution, rinsed with absolute ethanol and deionized water, and dried with nitrogen. The chip was then immediately mounted to the prism on the SPR sensor. The activation of carboxylic terminal groups was performed in situ by injecting deionized water followed by a 1:1 mixture of NHS and EDC for 5 min and deionized water again. Conditions for immobilization have been optimized in terms of running buffer composition and pH, as well as sufficient surface coverage. Immobilization of proteins via covalent attachment to COOH/OH SAM was performed at a flow rate of 30 μl/min and a temperature of 25 °C. To immobilize the receptors, sodium acetate (SA_{10}) pH 4.0 (Spα, N-SSC5D, and sCD6) or 5.0 (sCD5) was flowed through the activated surface until a baseline was achieved. Then, the SA_{10} solutions containing the receptors (2–5 μg/ml) were flowed across the activated surface until a desired surface coverage was achieved. To remove the non-covalently bound receptors, the high ionic strength PBNa (PBS**) buffer was

flowed along the sensor surface. Finally, the sensor surface was treated with 1 M EA to deactivate residual carboxylic groups.

Detection of the Interaction of the SRCR receptors with bacteria

Bacteria cells were pelleted from the culture media by centrifugation (4,000*g*, 5 min) and resuspended in PBS. To preserve bacterial cell morphology and to increase the sensitivity of the detection, cell aliquots were exposed to isopropanol (final concentration, 70% v/v) for 20 min at RT. The pellets of isopropanol-fixed cells were obtained by centrifugation at 7,000*g* for 5 min and washed twice with PBS.

To detect bacteria-protein interactions we used the following protocol: first a continuous flow of buffer (in the absence of bacteria), termed running buffer, was flowed along the sensor surface until a stable baseline was achieved. Next, bacteria resuspended in the running buffer at a concentration of 1×10^7 CFU/ml (or as indicated in the text) were delivered at a flow rate of 50 µl/min to the surface-immobilized proteins to allow the contact between the biomolecules and finally, the sensor surface was washed with running buffer (in the absence of bacteria) to dissociate receptor-bacteria complexes. Two different running buffers were tested for delivering the proteins, Tris and PBS. For *E. coli* the Tris buffer made better conditions for bacteria binding and for *L. monocytogenes*, the PBS was more suitable.

The binding of bacteria to the sensor surface was detected as the difference in the sensor response between the level obtained after the complete wash of the sensing surface after the flow of bacteria and the initial baseline level obtained before the injection of the bacteria solution (Fig. 2.5).

To account for potential artifacts that could affect SPR data analysis, we used reference-compensated measurements (Myszka, 1997; Ober and Ward, 1999; van der Merwe, 2001). Several different surfaces were tested to be used as a reference surface. These include a deactivated surface without ligands, surfaces covered with BSA, casein, NeutrAvidin or Streptavidin, and a surface with immobilized sCD5 (a protein known not to interact with bacteria). The study revealed that there was considerable adsorption of bacteria to bare alkylthiolate SAM (used as a functional layer) without any molecules immobilized and that the binding of bacteria to the surface coated with other molecules was significantly higher than that observed in case of

surface coated with sCD5. Therefore, one of the sensing channels was immobilized with sCD5 as reference channel.

2.3. Results

2.3.1. Detection of N-SSC5D binding to bacteria in conventional bacteria–protein binding assays

We first assessed the binding of the SRCR-containing extracellular domains of Spα, SSC5D, CD6, and CD5 (respectively, Spα, N-SSC5D, sCD6, and sCD5) to *E. coli* strains BL21(DE3), IHE3034, and RS218, and to *L. monocytogenes* strain EGD-e, using conventional bacteria-protein binding assays. Although Spα, sCD5, and sCD6 had previously been tested for bacteria binding, no experiments had been performed for SSC5D. We incubated 5 μg of each recombinant SRCR protein with bacterial suspensions of 1×10^8 live cells (colony-forming units, CFU) at 4 °C, followed by centrifugation and immunoblotting of the pelleted bacteria.

We confirmed the interaction of recombinant Spα with all bacterial samples tested, having an enhanced capacity to bind *E. coli* RS218 comparing with the other bacteria strains (Fig. 2.2). However, and in contrast with previous studies, no detectable sCD6 was recovered in association with the bacterial pellets, using our experimental setup. There was also no bacteria-bound sCD5 detected, but this was expected, given that CD5 was reported not to bind to bacteria (Vera et al. 2009). As observed from the experiments, N-SSC5D distinctly detected *E. coli* RS218 and IHE3034, although there was no conclusive evidence at this stage that it could bind to *E. coli* BL21(DE3*)* or to *L. monocytogenes*.

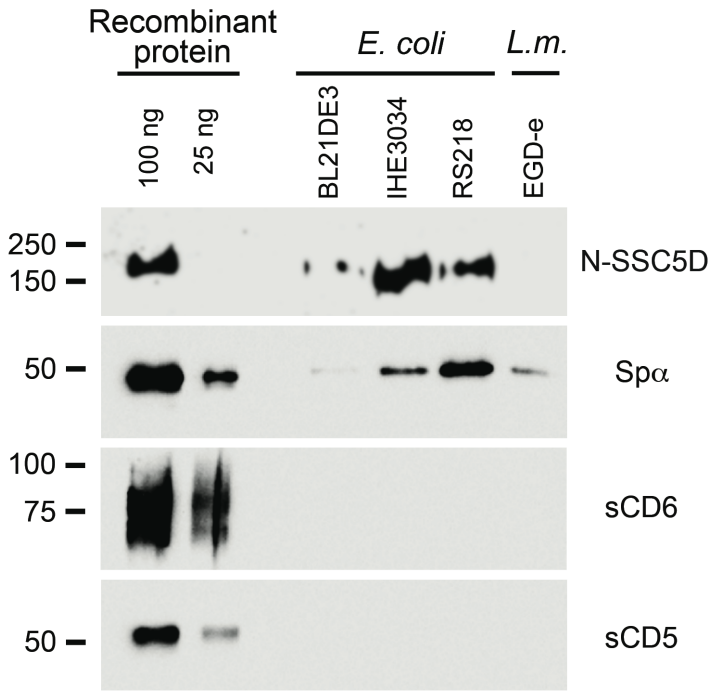

Figure 2.2. Spα and N-SSC5D bind bacteria. Recombinant Spα, N-SSC5D, sCD6, and sCD5 were incubated (5 µg each sample) with suspensions of 1 × 10^8 CFU of live *E. coli*, strains BL21(DE3), IHE3034, or RS218, or with *L. monocytogenes*, strain EGD-e. Cell-bound proteins were detected by immunoblotting using anti-HA mAb. Pure recombinant proteins were also run (100 and 25 ng, left lanes) to determine the sensitivity of the assay.

2.3.2. Use of SPR to probe bacteria binding

The results from the previous experiment suggested that different SRCR proteins had distinct binding properties to different bacterial strains, which might not have been highlighted in previous publications, each addressing a different SRCR protein at a time. Aware that western blot detection might not be the most sensitive method to emphasize these differences, we designed a new SPR-based assay to enhance the sensitivity of detection of extracellular proteins binding to bacteria.

A typical SPR experiment involves several tasks including ligand and analyte preparation (selection of buffers, concentration, purity, etc.), the selection of a sensor chip and ligand immobilization strategies, selection of a reference surface, analyte injection (checking for mass transfer limitation by using different flow rates), surface regeneration (if possible) and measurement of the sensor response and data analysis.

Immobilization of proteins via covalent attachment to a mixed self-assembled monolayer (SAM) of alkanethiols

Biosensing using SPR is highly dependent on the surface selected to immobilize the biomolecular recognition elements. The sensor surface should be properly covered with the biorecognition elements, while safeguarding their biological activity, and importantly, strategies to avoid non-specific adsorption to the surface must be anticipated (Homola, 2008). Self-Assembled Monolayers (SAMs) are organized unidirectional layers of molecules which spontaneously forms on a solid surface, producing a high-density background that can be regarded as the interface between metals, on one hand, and organic and biological materials such as polymers or biomolecules (peptides, antibodies, etc.) on the other (Homola, 2008). Due to their intrinsic characteristics, SAMs are easily prepared and are the most commonly used organic system to perform SPR-based assays with biological samples. In this thesis, we used a mixed SAM of alkanethiolates deposited on the chip gold layer. SAM surfaces constituted by alkanethiol molecules can be divided in three distinct parts (Love et al., 2005): 1) a sulphur group used to form a metal-thiol interaction (gold is often used because it is practically inert) (Nuzzo and Allara, 1983) 2) a spacer chain, typically made up of methylene groups $(CH_2)n$; n>10), that orient and stabilize the monolayer through Van der Waals forces, forming a crystalline structure (Bain et al., 1989) and 3) a functional head group that can be tailored to produce surfaces with different chemical properties (Wink et al., 1997). Alkanethiols terminated with carboxylic head groups were used to couple the proteins to the sensor chip via amino coupling. In amino coupling, the carboxylic groups of alkanethiols are activated with EDC/NHS forming activated esters, that react with the primary amines ($–NH_2$) of lysine residues to covalently couple proteins to the SAM (Fig. 2.3). A biosensor surface must also offer resistance to nonspecific adsorption of non-target molecules, especially proteins. A non-fouling background can be achieved by using a mixture of oligo(ethyleneglycol) terminated alkanethiols (Prime and Whitesides, 1993), where the hydroxylic group maintains a hydrophilic surface, which is known to significantly reduce protein adsorption.

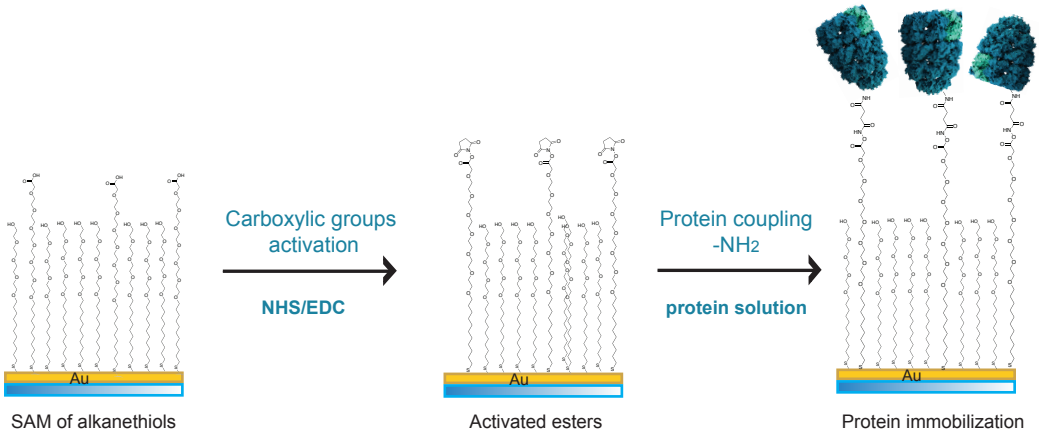

Figure 2.3. Schematic description of SPR chip surface functionalization and receptor immobilization. Here, a gold-coated glass plate was functionalized with a mixed SAM of alkanethiols that were used to covalently anchor the receptors by amino coupling and to form a stable non-fouling surface.

The nature of proteins has a crucial role in determining the best conditions for an appropriate immobilization After the activation step (EDC/NHS), the immobilization conditions for sCD5, sCD6, Spα and N-SSC5D were optimized regarding running buffer composition and pH, appropriate surface coverage and protection of the biological activity. Amino coupling is initially promoted by electrostatic interactions between the negatively charged carboxylic groups of SAMs and positively charged protein. To that end, proteins are diluted into a buffer with a low ionic strength buffer (to minimize charge screening) with a pH slightly lower than its isoelectric point or pI (to give the protein net positive charge) (van der Merwe, 2001). Accordingly, the theoretical isoelectric pIs of sCD5 (pI, 8.18), sCD6 (pI, 5.21), Spα (pI, 5.14) and N-SSC5D (pI, 6.11) were predicted from the amino-acid sequence using the ExPASy software (Gasteiger et al., 2003). We started by optimizing the immobilization conditions of sCD6 and sCD5 by diluting the proteins in 10 mM sodium acetate (SA_{10}) buffer pH 5 and observed the surface coverage (Fig. 2.4A). The SPR sensor response to the immobilized proteins should be about 7 nm (the shift in the resonant wavelength). In these conditions, a suitable immobilization level was achieved for sCD5; however, the level of immobilized sCD6 protein was very low, which was not unexpected because the pI of sCD6 was very close to the pH of the buffer used. Therefore, we tried more acidic conditions (pH 4) for sCD6, which resulted in an appropriate surface coverage (Fig. 2.4B). Similarly, to CD6, the coupling of Spα and N-SSC5D to the chip surface was found to be optimal when proteins were flowed in

(SA_{10}) buffer pH 4 (Fig. 2.4C, D). After the establishment of the immobilization conditions, we proceeded to protein-bacteria interaction analyses.

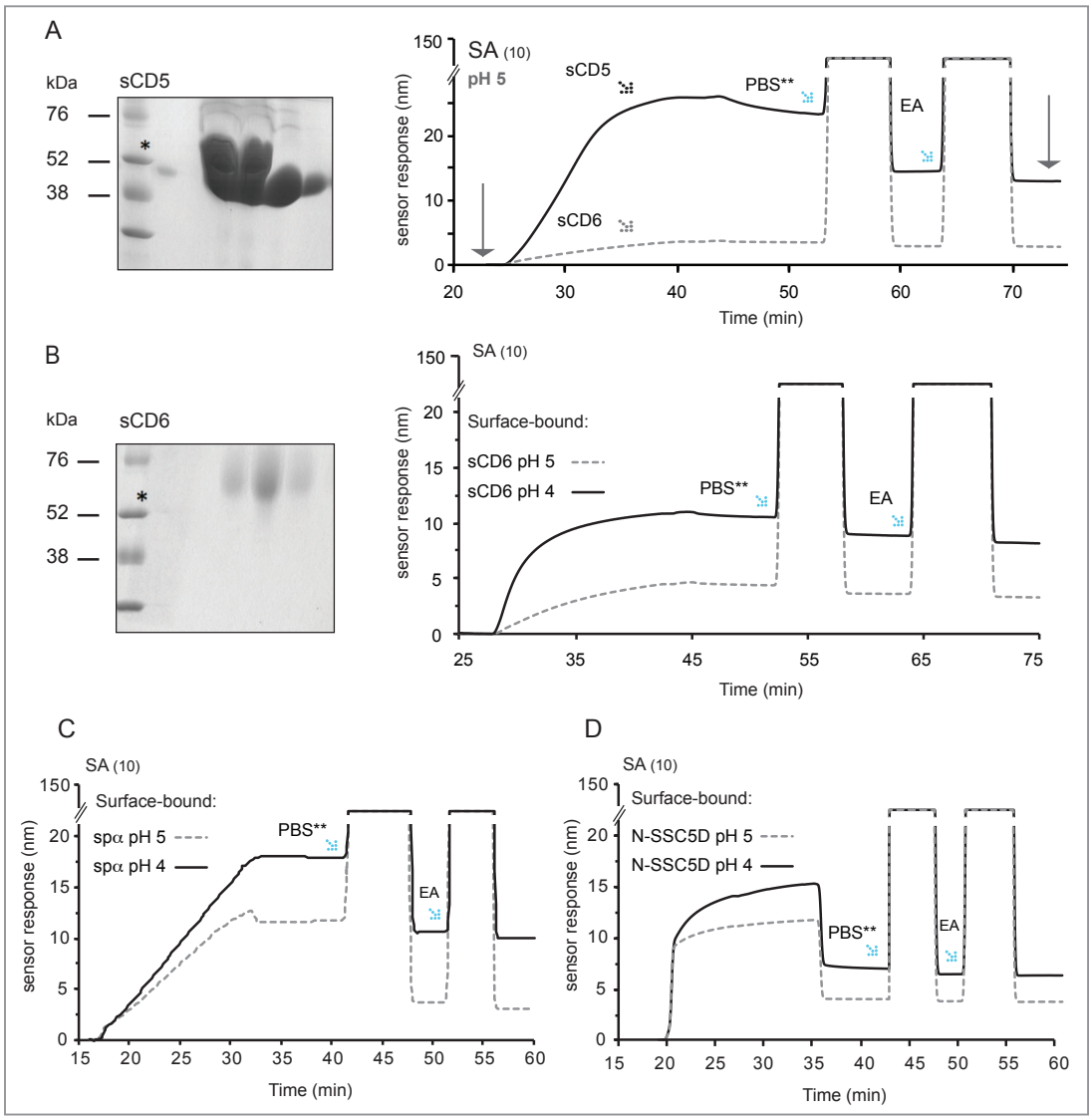

Figure 2.4. SPR sensor response to the immobilization of sCD5, sCD5, Spα and N-SSC5D using 10 mM sodium acetate (SA10) buffer with different pHs (4, 5). **A**. Coomassie staining of pure sCD5 (left) and sCD5 and sCD6 immobilization response using SA10 pH 5 (right). **B.** Coomassie staining of pure sCD6 (left) and immobilization response using SA10 buffer pH4 and 5. **C, D**. Evaluation of the Spα and N-SSC5D immobilization levels using SA10 buffer pH4 and 5. Following protein covalent coupling to the mixed SAM, high ionic strength PBS** buffer was flowed along the sensor surface to remove non-covalently bound protein, and ethanolamine (EA) was used to block the remaining activated sites to reduce non-specific binding. The sensor response is proportional to the quantity of the ligand bound to the surface.

N-SSC5D and Spα binding to bacteria is measurable by SPR

To confirm the suitability of our SPR approach to detect bacteria-receptor binding, Spα and sCD5 (reference channel) were immobilized in alternate flow chambers until a proper surface coverage was achieved. Next, suspensions of isopropanol-fixed bacteria, resuspended at a concentration of 1×10^7 CFU/ml were delivered to the sensor surface containing the immobilized proteins and SPR plots registered (Fig. 2.6A). The output of the SPR sensor (expressed in nanometer of resonant wavelength) is directly proportional to the amount of biomolecules attached to the surface of the sensor. The difference in the sensor output before the injection of bacteria (baseline level) and after washing the surface with the captured bacteria in running buffer is therefore proportional to the amount of bacteria captured (irreversibly bound) by the proteins immobilized on the sensor surface (Fig. 2.5). This quantity was used to characterize the ability of the respective immobilized proteins to bind bacteria.

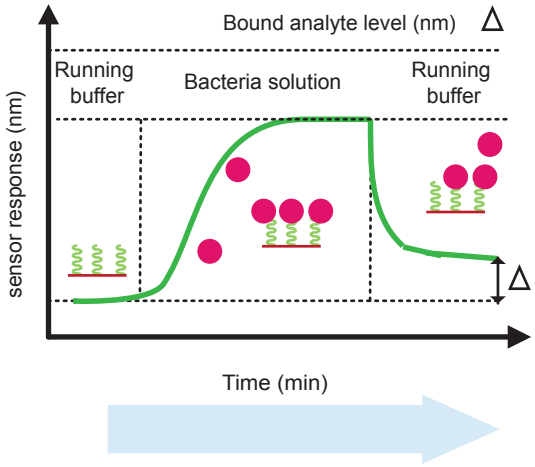

Figure 2.5. Sensor response to continuous measurement of analyte binding to immobilized receptors. Upon bacteria injection there is an increase in response due to protein-bacteria interaction. After the equilibrium is reached, running buffer is flowed over the surface, where a decrease in sensor response is observed due to the dissociation of the receptor-analyte complex. The amount of captured molecules Δ is determined as the difference between the level after the complete wash of the bound surface and the initial baseline sensor response of buffer (prior to bacteria injection).

Next, we tested whether the interactions of N-SSC5D with *E. coli* RS218 and *L. monocytogenes* EGD-e were measurable by SPR. We considered the following as references for the binding spectra: (a) the positive interaction of Spα with

neuropathogenic *E. coli* RS218 and with *L. monocytogenes* EGD-e and (b) the null interaction of sCD5 with both bacteria species.

As illustrated in Figure 2.6B, the interaction levels of N-SSC5D with bacteria were lower than those of Spα in both cases (between 15 and 40% across several experiments), but quite distinct from the profiles obtained for sCD5. These results confirmed the WB detection of the N-SSC5D-*E. coli* RS218 interactions seen in Figure 2.2, but further advanced in the detection of a subtle interaction between N-SSC5D with *L. monocytogenes*.

The results were reliable and qualitatively consistent among experiments, with only small variations in the absolute values of the responses. The chip-to-chip reproducibility of the interaction was >82% and >95% for N-SSC5D and sCD5 binding, respectively. The reproducibility values were determined from three independent experiments for each protein.

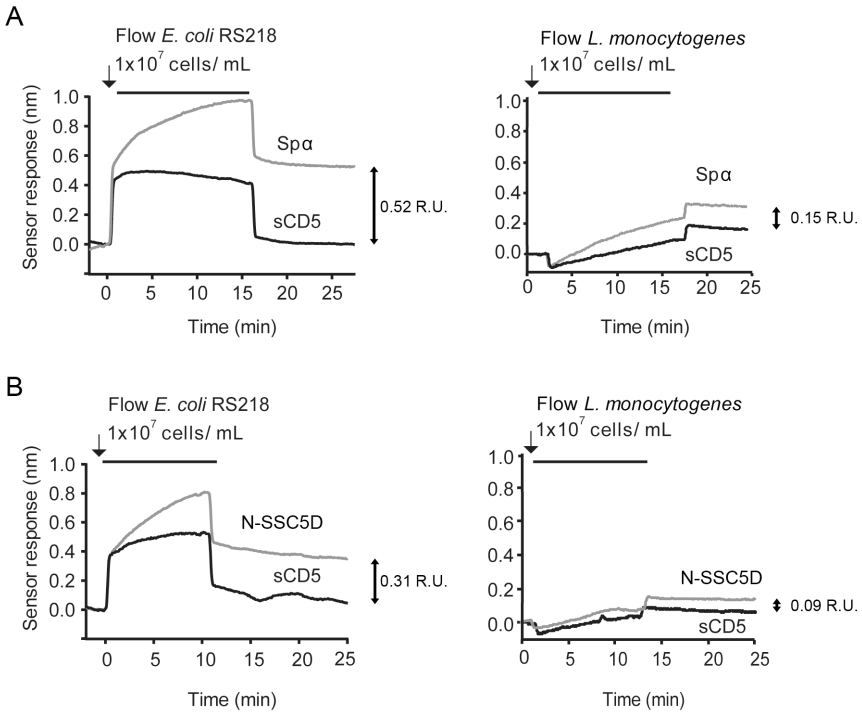

Figure 2.6. SPR detection of N-SSC5D binding to *E. coli* RS218 and *L. monocytogenes* EGD-e. Recombinant Spα **(A**) or N-SSC5D **(B)**, as well as the control sCD5 were immobilized in sensorchips and flowed with *E. coli* RS218 (left) *or L. monocytogenes* EGD-e (right) suspensions of 1 × 10^7 CFU/ml. After injection stopped, bacteria were retained in the different surfaces containing the SRCR proteins according to the strength of binding. Data are representative of multiple experiments with similar results.

N-SSC5D can distinguish between bacterial strains

To test whether N-SSC5D could have a different capacity to bind different *E. coli* strains, we immobilized N-SSC5D and simultaneously injected, in separate flow channels, the non-pathogenic laboratory BL21(DE3) strain, and the meningitis-causing RS218 and IHE3034 strains. As another control of null-binding, we used in the fourth flow channel, heat-killed IHE3034. In parallel, we performed the same experiment with immobilized Spα. As seen in Figure 2.7, *E. coli* RS218 gave the best binding curve to N-SSC5D, followed by IHE3034, and finally BL21(DE3). Heat-killed IHE3034 only marginally bound to N-SSC5D, suggesting that the bacterial determinants recognized by N-SSC5D are destroyed by heat. The binding profile of Spα to the different *E. coli* strains was not too different, binding marginally better to RS218 and BL21(DE3) than N-SSC5D, and less to IHE3034 than N-SSC5D, indicating that these proteins have slightly distinct recognition profiles but can nevertheless distinguish between different bacterial strains.

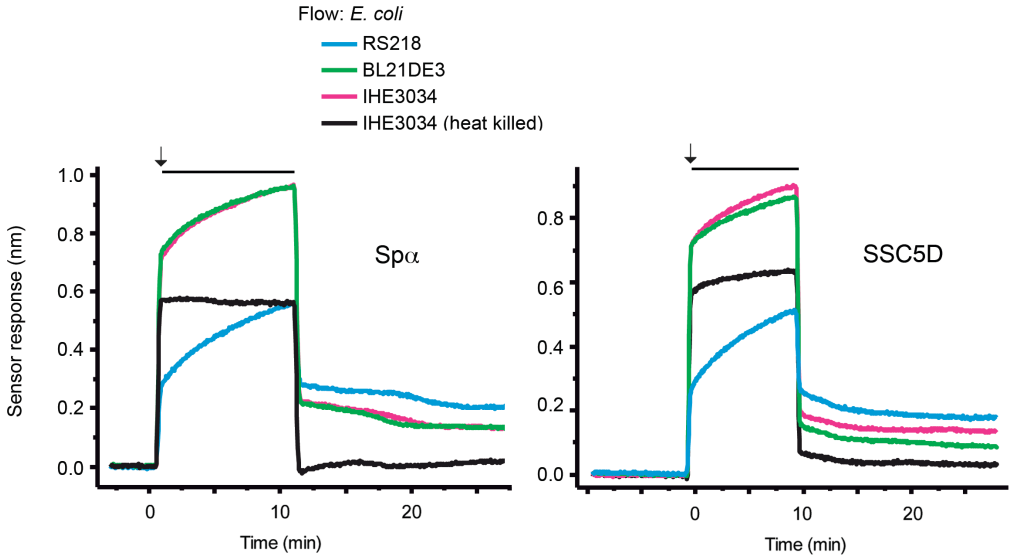

Figure 2.7. Temporal sensor response to the differential binding of N-SSC5D and Spα to different *E. coli* strains. Recombinant N-SSC5D (and Spα in parallel experiments) was immobilized in the four sensing channels and simultaneously injected suspensions of 1 × 10^7 CFU/ml of *E. coli* RS218, *E. coli* IHE3034, or *E. coli* BL21(DE3). The fourth flow channel was used to flow heat-killed IHE3034. After 10 min of injection, bacteria were differently retained in the four different sensor chambers. Data are representative of multiple experiments with similar results.

Differential binding of SRCR proteins to a same bacterial strain

To directly assess the differential binding capacity of the different SRCR receptors to a same bacterial preparation, we immobilized Spα, N-SSC5D, sCD6, and sCD5 in the four sensing channels and simultaneously injected *E. coli* RS218 at 1 × 10^7 CFU/ml to all channels. As depicted in Figure 2.8A, RS218 bound with the highest level to Spα, followed by N-SSC5D. As expected, sCD5 displayed the lowest level of RS218 binding; however, binding of the bacteria to immobilized sCD6 was, although relatively low, noticeably higher than that binding to sCD5. This indicates that despite the apparent negative result of Figure 2.2, there is some above-background level of binding of sCD6 to *E. coli* RS218 measurable by this SPR-based method.

Finally, we evaluated the sensitivity of the method by analysing the interaction of *E. coli* RS218 with N-SSC5D using suspensions with decreasing bacteria concentration. Figure 2.8B represents again the profiles of binding of *E. coli* RS218 at 1 × 10^7 CFU/ml to immobilized N-SSC5D and sCD5. Then, the specific binding was obtained by subtracting the signals arising from the measuring channels with immobilized N-SSC5D from those measured in the sCD5-immobilized reference channels. Three different concentrations of bacteria were used, 3, 5, and 10 × 10^6 CFU/ml, and for each concentration, the subtractive plots are represented in Figure 4C, indicating that the method clearly detects specific binding of *E. coli* RS218 to N-SSC5D even when using bacteria concentrations as low as 3 × 10^6 CFU/ml.

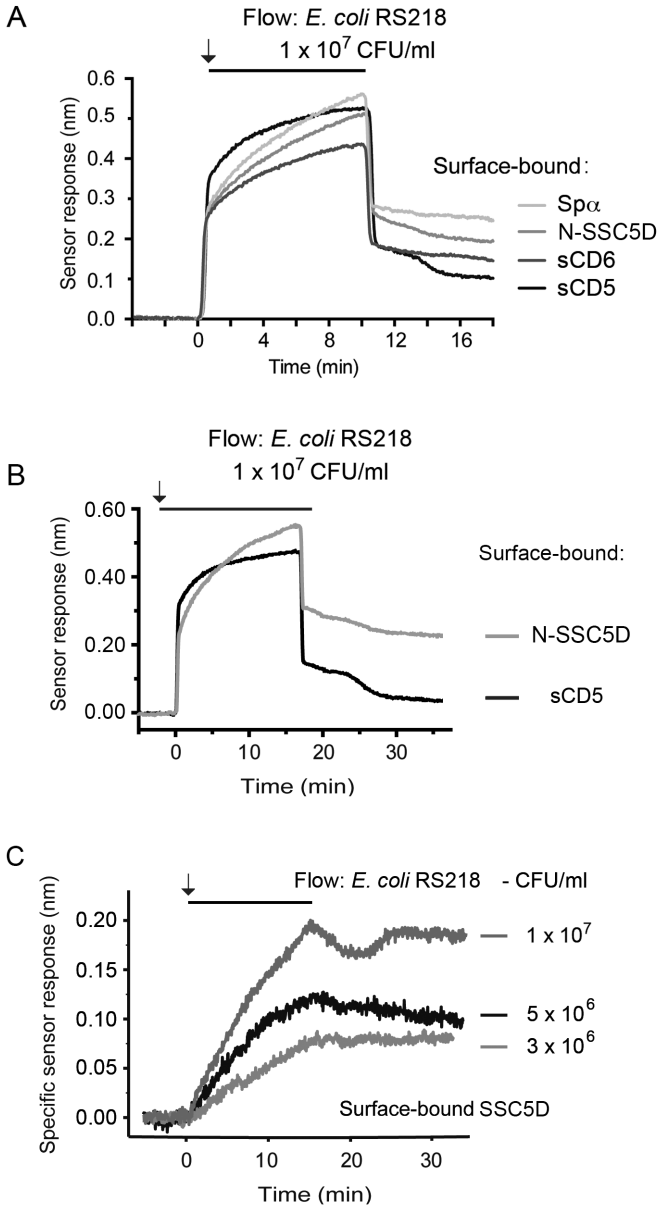

Figure 2.8. Temporal sensor response to the binding of SRCR proteins to *E. coli* RS218. **A.** Recombinant Spα, N-SSC5D, sCD6, and sCD5 were immobilized in the four sensing channels and simultaneously injected with an *E. coli* RS218 suspension of 1 × 10^7 CFU/ml. **B.** Recombinant N-SSC5D and sCD5 were immobilized on alternate chambers, and *E. coli* RS218 was flowed at 1 × 10^7 CFU/ml. **C.** The specific binding of E. coli RS218 to N-SSC5D was obtained by the subtraction of the non-specific response registered for sCD5 from the measured signals of *E. coli* RS218 binding to N-SSC5D, for bacterial concentrations of 3, 5, and 10 × 10^6 CFU/ml.

2.4. Discussion

The SRCR-B family comprises a group of proteins that have a very high level of genetic conservation and remarkable structural similarity of the SRCR domains. However, each member has been described with very exclusive functions, as diverse as roles in signal transduction, regulation of inflammation, cell survival and apoptosis, differentiation, detoxification in iron metabolism, to name just a few, to such an extent that the structural properties of the SRCR modules may be so far the only proven unifying feature of the family. This diversity in functions can be in part explained by the fact that each protein has unique features (different number of SRCR domains), is expressed in different contexts and architectures (membrane bound in different cell types, carrying cytoplasmic domains of variable lengths and compositions, or is secreted), may have additional domains of other types, and can display different degrees of posttranslational modifications, such as *O*- and/or *N*-glycosylation.

Recently, the description of a physical interaction between Spα, which is a small soluble protein almost exclusively composed of the three SRCR domains, and several strains of bacteria (Sarrias et al., 2005) projected an explicit PRR function for such type of domain. Similar microbe-binding properties of other SRCR proteins have indeed been assigned to their own SRCR domains (Prakobphol et al., 2000; Sarrias et al., 2007; Fabriek et al., 2009). To further explore this possible unifying role for SRCR domains, we thus investigated the PRR-type properties of the recently described protein SSC5D, and more specifically of its N-terminal SRCR-containing moiety. For this purpose, we designed an SPR-based assay for rapid, direct and real-time observation of immune receptor–bacteria binding events.

Conventional methods used previously to assay the interaction of bacteria with secreted recombinant SRCR (or other) proteins, such as flow cytometry or immunoblotting, rely on the labelling of proteins with a fluorescent dye, such as FITC (Kneidl et al., 2012), or with biotin targeting the sulfhydryl groups of cysteine residues (Sarrias et al., 2005; Sarrias et al., 2007; Vera et al., 2009). Among the many practical advantages of the SPR method compared with conventional ones, there is no requirement for receptor labelling, and only minute amounts of protein are needed to generate distinct or differential signals. In our conventional assays shown in Figure 2.2, we used 5 μg of recombinant protein and 1×10^8 CFU per individual receptor–bacteria assay, and some of these interactions were on the borderline of western blot sensitivity. By comparison, 2 μg of recombinant protein could be used in a single SPR

assay testing the interaction with up to four bacteria types, these also used at smaller amounts (typically at 1×10^7 CFU/ml, but feasibly down to 3×10^6 CFU/ml), which represent an improvement of the detection of protein–bacteria interactions. Moreover, the versatility of our setup allows having up to four different immobilized proteins and simultaneously comparing the binding of each protein to the same bacterial suspension as analyte, or conversely, comparing directly in the same assay suspensions of four different bacteria binding to the same immobilized protein.

Surface plasmon resonance biosensor technology-based affinity and kinetic measurements are typically performed with analytes that are monovalent (van der Merwe, 2001). Although through complex analyses it is possible to obtain such parameters in the case of multivalent (bacterial) contacts (Chung et al., 2007), here we have utilized SPR to detect interaction *per se* and to make synchronized measurements, obtaining direct comparable data for sets of four different receptors, or four different bacteria samples. It should be noted that we chose to consider the amount of captured (irreversibly bound) bacteria to characterize the ability of the respective proteins to bind selected bacteria, as the reported experiments with bacteria are complex, and the binding curves in response to bacteria are not determined only by kinetic parameters of the interactions; they are also affected by other factors, such as background refractive index changes (due to differences in the composition of samples containing bacteria and running buffer), the non-specific adsorption of bacteria onto the sensing surface, and especially by mass transport limitations (due to rather slow diffusion of bacteria to the sensing surface) (Myszka, 1997; Ober and Ward, 1999; van der Merwe, 2001).

An important aspect in the design of the assay is the choice of a reference, which allows for the compensation of changes in the refractive index due to unspecific events. In the context of our study, sCD5 was defined as such based on the literature and on the result obtained with our conventional assay. Additionally, we chose to use sCD5 in experiments, as this protein is genetically and structurally related with the query molecules N-SSC5D and Spα, and thus it would account for intrinsic unspecific binding features that can be particular to the SRCR family of molecules.

From the experiments described in the present work, we show for the first time that, like some other human SRCR proteins, SSC5D, through its set of SRCR domains, has the capacity to bind bacteria and, from the direct comparisons established using the multichannel SPR apparatus, that N-SSC5D and Spα can distinguish between different types of bacteria on one hand and different strains of one type of bacteria on the other.

Binding of N-SSC5D and Spα to *E. coli* RS218 gave higher sensor responses than binding to BL21(DE3). While BL21(DE3) is a well-characterized non-pathogenic research model commonly used in academic laboratories and in the biotech industry, RS218 is a pathogenic strain belonging to the serotype O18ac:H7:K1 and displaying virulence factors that contribute to the onset of meningitis. The IHE3034 strain also belongs to the same serotype and although N-SSC5D binds better to IHE3034 than to BL21(DE3), the same behaviour is not observed for Spα, suggesting that SRCR proteins may have very defined discriminatory properties on different, still undefined, extracellular components of bacteria. Likewise, the response signals for N-SSC5D and Spα binding to *L. monocytogenes* were significantly lower than to *E. coli*, possibly reflecting a differential sensing of Gram-positive vs. Gram-negative bacteria, but at this stage and with very few bacteria types tested, it is premature to establish any categorization.

The interactions of N-SSC5D and Spα with *E. coli* RS281 were relatively strong and specific and, as shown for N-SSC5D, the sensor responses increased proportionally to the concentration of the bacterial suspensions used. Comparing with the conventional assays, binding to *E. coli* IHE3034, also a meningitis-causing pathogen, did not give the same precise results, as N-SSC5D bound less and Spα bound better in the SPR experiments than in the bacteria-binding assays. SPR offers substantial benefits when compared with these methods, because it allows real-time detection of bacteria and, moreover, since bacteria are delivered under conditions of continuous hydrodynamic flow, the SPR technique is expected to better mimic the protein–bacteria interaction under physiological conditions where shear forces promoted by the body fluids are likely present (Bustanji et al., 2003; Salminen et al., 2007). As measurements are obtained simultaneously for the different proteins/bacteria within the same experiment, we can be confident that they truly reflect quantitative differences in binding of SRCR proteins to bacteria.

CD6, on the other hand, was reported to bind to Gram-positive and Gram-negative bacterial strains (Sarrias et al., 2007). CD6 is a receptor of T lymphocytes that has characterized roles in the regulation of T cell signalling and in inflammatory responses (Oliveira et al., 2012; Pinto and Carmo, 2013), so its role as a pathogen sensor was unexpected. From the results of our conventional assay shown in Figure 2.2, we would have concluded that either sCD6 does not bind to the tested bacteria or that it binds with such low affinity that the interaction does not survive the pelleting and washing of the bacteria. However, our improved SPR assays may highlight a slightly different

conclusion: although the level of binding of sCD6 to *E. coli* RS218 (Fig. 2.8) was significantly lower than that of either N-SSC5D or Spα, it stayed clearly above the level of the sCD5 negative profile. Apart from the higher sensitivity over the previous methods, SPR is run at the more adequate temperature of 25 °C, whereas conventional protein–bacteria binding assays are customarily performed at 4 °C. Notwithstanding the fact that the bacteria-binding capacities of sCD6 are reduced comparing with N-SSC5D or Spα, it is nonetheless very plausible that sCD6 may have true microbe-sensing properties, which are highlighted by its capacity to protect animals from LPS-induced septic shock and in a lethal model of polymicrobial sepsis (Sarrias et al., 2007; Martínez-Florensa et al., 2017).

In conclusion, we have demonstrated through the use of a dynamic, antibody-free, SPR-based assay that N-SSC5D, like Spα, is capable to physically interact with whole bacteria cells. This new approach can be adapted to screen for interactions with a wide range of bacteria and once the best bacterial targets of N-SSC5D are identified, this will hopefully allow to better characterize and more deeply explore the role of this SRCR protein in pathogen sensing and in driving immune responses. The results obtained in this study using the SRCR-containing moiety of SSC5D will undoubtedly further our understanding of the specific function of SRCR domains as the functional parts of a family of mammalian proteins that have enhanced capabilities to recognize and eventually fight bacterial pathogens.

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Chapter 3

Tissue localization and expression of SSC5D

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3.1. Introduction

In the previous chapter, we observed that N-SSC5D could bind and discriminate between different strains of bacteria. This capacity is essential in certain body locations such as intestine, lung and genitourinary tract where PRR, albeit being in permanent contact with beneficial resident microbiota, can also contact invading virulent microorganisms that have to be effectively neutralized. Mucosal tissues are therefore well-equipped with both innate and adaptive immune components, and its defence is highly dependent on the PRR expressed not only by immune cells, but also by the epithelia (Tlaskalová-Hogenová et al., 2011). Previous results revealed that the SSC5D gene is expressed in the **placenta**, **spleen** and more weakly in the **colon** and **lung** (Gonçalves et al., 2009), which suggests a biological function in placental and mucosal immunity.

Trophoblast cells are the main structural and functional components of placenta. These are implicated in the maternal-foetal interface formation (Red-Horse et al., 2004), which allows the exchange of oxygen and nutrients, and has also an important immunomodulatory role (Mor and Cardenas, 2010; Mor and Kwon, 2015). Trophoblasts directly interact with maternal decidual immune cells, including NK cells, macrophages and regulatory T cells, which direct placentation and regulate maternal-foetal tolerance (Munoz-Suano et al., 2011). In addition, the trophoblast layer is also important to resolve uterine infections that often result in pregnancy complications such as preterm delivery, abortion or preeclampsia (Koga et al., 2009; Mor and Cardenas, 2010). To protect the embryo from the detrimental effects of pathogenic agents, trophoblasts work as innate immune cells through the expression of PRR that sense not only virulent agents but also host damage signals (Koga et al., 2009; Mor and Cardenas, 2010). Trophoblasts cooperates with maternal immune cells to mediate immune responses and their PRR include TLR and cytoplasmic-based NLR (Abrahams, 2011).

Interestingly, the expression profile of TLR and NLR varies according to the placenta development phase; and the nature of each immune response is influenced by the type of stimuli perceived, which can derive mild inflammatory responses to severe cell apoptosis (Koga et al., 2009; Abrahams, 2011; Pudney et al., 2016). Accordingly, a successful pregnancy upon microbial challenge is intrinsically related to an adequate innate trophoblast immune response (Mor and Cardenas, 2010), for instance elevated levels of the inflammatory cytokine IL-1β may trigger prematurity (Abrahams, 2011). In addition, the trophoblast is itself capable to produce anti-microbial peptides such as the

secretory leukocyte protease inhibitor (SLPI), a major anti-viral factor (McNeely et al., 1997; Sallenave, 2002). Interestingly, a study proposed the existence of a low abundant commensal 'placenta microbiome' (Aagaard et al., 2014). Nevertheless, owing to the complexity of the studies required to further this matter, the existence of a placental microbiota is still a controversial issue (Hornef and Penders, 2017).

As above-mentioned, mucosal tissues, including the intestine where SSC5D is expressed, are rich in PRR expressed by immune and epithelial cells. However, so far the evaluation of the expression of SSC5D expression has been limited to the mRNA analysis of immune derived cell lines and peripheral blood monocular cells. Therefore, we sought to investigate SSC5D protein expression in cells from diverse origins, using a novel antibody, and to evaluate its distribution in different parts of biological tissues.

Moreover, to fully understand the physiological importance of SSC5D, an important part of our proposed studies included the generation of SSC5D-deficient mice. As already mentioned in the general introduction, KO mice have been essential to the acknowledgment of TLR as PRR, and to predict some of the functions of the SRCR members, such as the anti-apoptotic role of Spα (Miyazaki et al., 1999). Hence, *in vivo* studies in mice depleted of SSC5D should help to clarify its putative functions, and in particular its role as a PRR and in mucosal homeostasis.

3.2. Material and Methods

Cell lines

Cell lines JTAg, E6.1, K562, KG-1, Raji and THP-1 were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% dialyzed foetal calf serum (FCS) (First Link), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a 5% CO_2 humidified incubator. RPMI 1640 and all supplements were obtained from (Gibco, Life Technologies) except for FCS (First Link). Cell lines A498, A549, ACHN, Caco-2, HeLa, MCF-7 and TCCSUP were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (20% TPC-1) FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a 5% CO_2 humidified incubator. DMEM and all supplements were obtained from (Gibco, Life Technologies) except for FCS (First Link).

Peripheral blood mononuclear cells isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult blood donors from Hospital de São João (Porto, Portugal) by density gradient centrifugation using Lympholite®-H (Cedarlane). Erythrocytes were lysed by using a red blood cell lysis buffer (0.01 M Tris, 0.15 M NH_4Cl, pH 7.2) for 8 min at 37 ºC. Monocytes were separated from peripheral blood lymphocyte by adherence for 1 h at 37 °C in a 5% CO_2 humidified incubator. Monocytes were differentiated into macrophages with macrophage colony-stimulating factor (M-CSF) at 50 ng/ml (Immuno Tools) and to dendritic cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immuno Tools) and IL-4 (Immuno Tools) at 25 ng/ml. Cells were maintained in culture for 7 days in RPMI- 640 medium supplemented with 10% FCS 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in a 5% CO_2 humidified incubator.

Cell lysis

Cells at a density of 50 × 10^6 cells/ml were used to prepare the lysates. For adherent cells (80% confluence), medium was discarded and cells were washed with ice-cold PBS and detached by scrapping. Then, both adherent cells and suspension cells were collected by centrifugation at 450*g* for 5 min and washed twice with ice-cold PBS twice. The supernatant was discarded and cells were treated with lysis buffer (1% Triton X-100, 1 mM PMSF) on ice for 15 min. Next, cell lysates were transferred for microcentrifuge tubes and centrifuged at 20,000*g* at 4°C for 10 min. The supernatant was collected and the pellet was discarded. To perform the immunoblotting, the supernatant was diluted (1:1) in Laemmli Sample Buffer (Bio-Rad) with β-mercaptoethanol at 10% and boiled at 95 ºC for 5 min, then 20 µl were loaded into the gel.

Western blotting analysis of SSC5D

Cell lysates were run in SDS- PAGE for 1 h at 150 V with Tris/glycine/SDS running buffer (Bio-Rad). Samples were transferred to the nitrocellulose membrane using the iBlotTM Gel Transfer Device (Invitrogen) following the manufacturer's instructions. Then, the membrane was blocked with TBS, 0.1% Tween 20 (TBS-T), containing 5%

non-fat dried milk, for 1 h with shaking. SSC5D was detected with rabbit anti-SSC5D RB36409 (Abgent, 1:500) and in TBS-T with 3% non-fat dried milk, for 1 h at RT, followed by peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma, 1:30,000) for 1 h at RT. Immunoblot was developed using Amersham ECL detection reagent (GE Healthcare Life Sciences) and exposed to BioMax MR films (Kodak). The protein α-tubulin was used as a loading control and was detected with anti-α-tubulin (Sigma, 1:10,000) followed by goat anti-mouse-HRP (Santa Cruz, 1:20,000).

Immunohistochemistry (IHC) protocol

Human tissues for IHC were obtained from Hospital Pedro Hispano with the local ethical approval. For IHC assays, 4 µm sections from neutral buffered formaldehyde-fixed paraffin-embedded human tissue were deparaffinised in xylene, hydrated in an ethanol gradient (100%, 96%, 70%) until water. Antigens were retrieved with 10 mM sodium citrate pH 9 for 30 min at 96 ºC. After heating, slides were left in buffer for 15 min at RT. The following steps for the immunostaining were done using the kit Ultra Vision LP Value Detection System – HRP Polymer and DAB Chromogen (Dako). Primary polyclonal antibody rabbit anti-SSC5D (Abgent) was incubated for 1 h at RT (1:500 dilution in Ab Diluent S2022; Dako). Sections were counterstained in Mayer's haematoxylin, dehydrated using graded alcohols (70%, 96% and 100%) and xylene. Finally, cover slips were mounted with DPX (Sigma). No immunostaining was observed when the primary antibody was omitted or substituted with a pre immune rabbit serum.

Flow cytometry staining protocol

Cells were harvested by centrifugation at 450*g*, 5 min, 4 ºC and adjusted to 1×10^6 cell/ml. Then, cells were washed twice with ice cold FACS Buffer (PBS, 2% FCS, 0.05% NaN_3). For surface staining, anti-SSC5D was diluted in FACS Buffer (1:100) and cells were incubated for 30 min on ice, in dark conditions. Cells were then washed twice and incubated with anti-rabbit-PE (1:100, Invitrogen) 30 min on ice, dark conditions. After cell 2-step washing, cells were fixed with fixing buffer (PBS, 1% paraformaldehyde) 15 min at RT, washed twice and resuspended in PBS buffer. Cells were analysed in a FACS Calibur I flow cytometer (BD Immunocytometry Systems). Data were analysed using the FlowJoTM software (Tree Star). For intracellular staining (ICS), cells were fixed with fixing buffer (PBS, 1% paraformaldehyde) for 15 min at RT

and washed twice with FACS buffer. Then, anti-SSC5D (Abgent) was diluted in ICS Buffer (PBS, 2% FCS, 0.4% saponin, 0.05% NaN_3) (1:100), and cells were incubated for 30 min on ice, dark conditions. Cells were then washed twice and incubated with anti-rabbit-PE diluted in ICS buffer (1:100) for 30 min on ice, dark conditions. Cells were washed twice with FACS buffer, resuspended in PBS and analysed in a FACS Calibur I flow cytometer (BD Immunocytometry Systems). Data were analysed using FlowJoTM software (Tree Star). Isotype control (IgG) rabbit SC 2027 (Santa Cruz).

Confocal microscopy analyses of Caco-2 cells

Cells were seeded at density of 1×10^5 in µ-Dish 35 mm ibiTreat surface (ibidi) overnight in DMEM, supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a 5% CO_2 humidified incubator. On the following day, cells were washed twice with PBS and fixed with 3.7% paraformaldehyde for 15 min. Then, cells were washed with PBS and incubated for 30 min with buffer (PBS, Triton 0.1%, BSA 1%). Anti-SSC5D (Abgent) was diluted in buffer (1:500), and cells were incubated for 1 h on ice, dark conditions. Cells were then washed twice with PBS, 0.1% Triton. Then, goat anti-rabbit conjugated Alexa Fluor® 647 (Life technology) was diluted (1:500) in buffer and cells were incubated for 30 min on ice, dark conditions. Cells were washed twice with PBS. Nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min (1:1000, Molecular Probes). Finally, one drop of VECTASHIELD® Mounting Media (Vector Laboratories) was delivered onto the cells to preserve fluorescence. Cells were analysed with Laser Scanning confocal microscope Leica TCS SP5 II (Leica Microsystems, Germany).

Targeting vector construction

To create the 5' homology arm of the construct to delete mouse *Ssc5D*, we needed to introduce 1) consensus 34 base pair DNA recognition sites (*loxP* sites) located at 600 bp upstream the start codon of *Ssc5d* and 2) to amplify the genomic region corresponding to exons 1-5. To insert the *loxP* site, we performed a chimera PCR with two fragments of mice genomic DNA To amplify the first fragment (900 bp), which included a *Not*I restriction site upstream *loxP* and part of the *loxP* site we performed a PCR using the sense primer 5´-CTGGCGGCCGCTAGAGACCATGCCCACTGGATAG-

3´ (*Not*I site underlined) and anti-sense 5´-CGTATAGCATACATTATACGAAGTTATTTATTACCAGTTCCCAGAACAAAG-3´ in a 50 µl mixture containing 1 µM of each primer, 250 ng of mice genomic DNA, 10 mM dNTPs, 0.2 µl $MgCl_2$ and 1 U of phusion enzyme (FINNZYMES) with the following cycling conditions: 98 ^{o}C for 30 s and 25 cycles of 98 ^{o}C for 10 s, 65 ^{o}C for 20 s and 72 ºC for 30 s, followed by a final extension of 72 ºC for 7 min. Next, we performed a second two-step PCR to insert the *Sma*I restriction site (2800 bp) using the following primers 5´-CTTCGTATAATGTATGCTATACGAAGTTATGTTCAGACACTCTTTTCTCGAATC-3´ and 5´-CTACCCGGGATCTGAAGGGGCACTGTGAACGC-3´ (*Sma*I site underlined) in a 50 µl mixture including 1.5 µM of each primer, 250 ng mice genomic DNA, 10 mM dNTPs and 2.6 U of Expand High Fidelity PCR System (Roche) with the following cycling conditions: 94 ^{o}C for 2 min, 10 cycles of 94 ^{o}C for 15 s, 65 ºC for 30 s and 68 ^{o}C for 2.5 min, followed by additional cycling conditions 20 cycles of 94 ^{o}C for 15 s, 65 ºC for 30 s and 72 ^{o}C for 2.5 min and final extension of 72 ºC for 7 min. The two fragments (*Not*I-*loxP*) and (*loxP*-*Sma*I) were purified with GeneClean kit (MP Biomedicals) and the total 5' homology (3.7 kb) arm was amplified using 2 µM of 5´-CTGGCGGCCGCTAGAGACCATGCCCACTGGATAG-3´ (*Not*I site underlined) and 5´-CTACCCGGGATCTGAAGGGGCACTGTGAACGC-3´ (*Sma*I site underline) primers, 10 mM dNTPs and 2.6 U of Expand High Fidelity PCR System (Roche) with the following cycling conditions: 94 ^{o}C for 2 min, 10 cycles of 94 ^{o}C for 15 s, 65 ºC for 30 s and 68 ^{o}C for 4 min, followed by additional cycling conditions 20 cycles of 94 ^{o}C for 15 s, 68 ºC for 30 s and 72 ^{o}C for 4 min and final extension of 72 ºC for 7 min. The 5-homology arm was subsequently purified with the GeneClean kit (MP Biomedicals), digested with *Not*I and *Sma*I and cloned into the–*Not*I/*Sma*I digested mammalian expression, Cre/Lox PGKneoF2L2DTA vector (Adgene). To amplify the 3' homology arm (4 kb) corresponding to the genomic region between exons 6-8 including *Sal*I and *Hind*III restriction sites, we used the following primers 5´-GCTGTCGACCAGTCAGGGCTGGCCACCTCC-3´ (*Sal*I underlined) and 5´-AGTAAGCTTTGTCCAGGCAGGCAGCTGTCTCC-3' (*Hind*III underlined) in a 50 µl mixture including 2 µM of each primer, 250 ng mice genomic DNA, 10 mM dNTPs and 2.6 U of Expand High Fidelity PCR System (Roche) with the following cycling conditions: 94 ^{o}C for 2 min, 10 cycles of 94 ^{o}C for 15 s, 60 ºC for 30 s and 68 ^{o}C for 2.5 min, followed by additional cycling conditions 20 cycles of 94 ^{o}C for 15 s, 60 ºC for 30 s and 72 ^{o}C for 2.5 min and final extension of 72 ºC for 7 min. The amplified fragment was subsequently purified with the GeneClean kit (MP Biomedicals), digested with *Sal*I and *Hind*III and cloned into the *Sal*I/*Hind*III digested PGKneoF2L2DTA vector.

Electroporation and selection of ES cells

After cloning the *Ssc5d* fragments into the PGKneoF2L2DTA vector, we linearized the DNA construct (restriction enzyme) to obtain a targeting vector. For the electroporation of CJ7 mouse embryonic stem cells (Swiatek and Gridley, 1993), we used 25 ug of the previously linearized DNA, purified by phenol:chloroform extraction, ethanol precipitated and resuspended in 50 µl of PBS. A single cell suspension of 10 x 10^6 cells from an 80% confluent culture was obtained by trypsinization, then washed twice with PBS at room temperature and resuspended in 800 ul of PBS. Afterwards, the cell suspension was mixed with the DNA and loaded onto an electroporation cuvette (0.4 cm gap). Electroporation was performed with a pulse at 0,24 kV, 500 uF. Cells were then removed from the cuvette, diluted into 25 ml of complete ES cell medium (Robertson, 1989) (without selection) and distributed into five 60 mm plates, coated with feeder cells (Stewart et al., 1992). Selection was done with 350 µg/ml of G418 (Geneticin, an analog of neomycin) added to the complete ES cell medium. Selection started 24 hours after electroporation and was maintained for 6 days (the G418-containing medium was changed every day), then individual colonies were picked for further analysis.

Cloning and labelling of KO probe NAT14

The genomic DNA fragment to be used as probe (*NAT14*) was amplified in a 50 µl mixture including 300 nM of the sense primer 5'- GCTCTGACACGACCTCCA -3' and 300 nM of the anti-sense 5'-TTGCTGAATTCCCTAACCAG-3´, 250 ng mouse genomic DNA, 10 mM dNTPs and 2.6 U of Expand High Fidelity PCR System (Roche) with the following cycling conditions: 94 ^{o}C for 2 min, 30 cycles of 94 ^{o}C for 15 s, 55 ºC for 30 s and 72 ^{o}C for 45 min, followed by a final extension of 72 ºC for 7 min. The amplified product corresponding to a band of 500 bp was purified using GeneClean kit (MP Biomedicals) and 4 µl of purified amplicon was inserted into p-GEMT (T4 DNA ligase - Roche).

To label the probe we repeated the PCR and added 4 µl of the PCR reaction to 41 µl of dd$H_2$0, then the DNA probe was heated for 5 min at 98 ºC and then rapidly cooled in ice to prevent renaturation of the complementary strands. The probe was purified with Illustra NICK columns (GE health care). The column was first washed with 2.5 mL

Tris/EDTA buffer (TE) and then equilibrated with 2.5 mL TE. The DNA probe was eluted with 50 µl of TE buffer. In the radioactive safe room, the probe was labelled with [α-^{32}P]dCTP using Redi prime II DNA labelling system (Amersham) and incubated for 1 h at 37 ºC. To purify the radioactive probe, the Illustra NICK column was first washed with 400 µl of TE and then the probe was eluted with 400 µL of TE. The probe was next heated at 99 ºC for 5 min and then rapidly cooled in ice. Next, the 400 µL of TE containing the probe were added to the membrane with the digested DNA clones and incubated under rotation (biometra ov 3) overnight at 65 ºC to hybridize with DNA. This probe hybridizes specifically with the last exon of *NAT14*, the gene preceding *SSC5D*.

Southern-blotting

Fifty-tree neomycin-resistant clones were selected and digested with *Hind*III and *Spe*I or *Spl*I. Next, the digested DNA was run on an 0.8% agarose gel. The gel was treated with 1) 0,25 M HCl 20 min – to depurinate the DNA fragments to improve the transference 2) 0.5 M NaOH/ 1.5 M NaCl for 45 min to denature the double-stranded DNA and 3) 0.5M Tris 7.5/3 M NaCl for 45 min to neutralize the gel. Then, the DNA was transferred from the gel to the nylon membrane (Bio-Rad #162-0196 zeta-Probe (30 x 3,3)) and treated according to the manufacturer's instructions.

3.3. Results

The SSC5D protein is widely expressed in cells from diverse origins

Protein expression was evaluated by WB using a novel antibody anti-SSC5D and immortalized cell lines derived from kidney (A498, ACHN), bladder (TCCSUP), lung (A549), colon (Caco-2), cervix (HeLa), breast (MCF-7), thyroid (TPC-1); and from immune cells, T cell (E61), B cell (Raji), monocytic (THP-1), and lymphoblast-like (K562) and myeloblast-like myelogenous leukemia cell lines (KG-1) (Fig. 3.1)

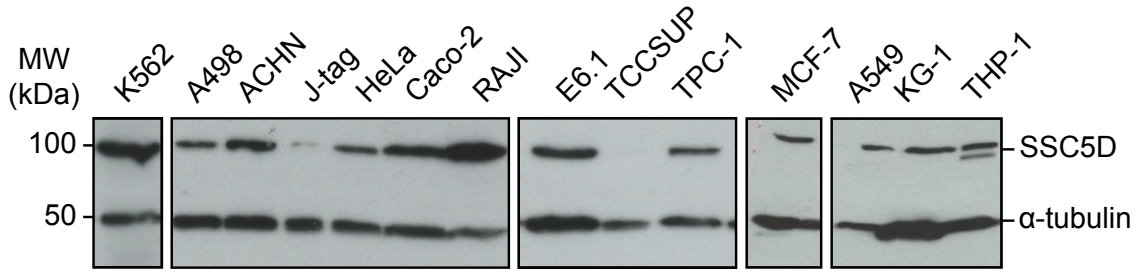

Figure 3.1. SDS-PAGE (7.5%) immunoblotting of the cell lines lysates using the anti-SSC5D antibody followed by peroxidase-conjugated goat anti-rabbit IgG antibody.

The WB analysis of SSC5D revealed a broad expression in several types of cells. Interestingly, SSC5D is found in cells derived from mucous tissues, including lung and gastrointestinal tract, and in kidney, where mouse *Ssc5d* was shown to be highly expressed (Miró-Julià et al., 2014). All the cell lines presented a band of ~100 kDa with the exception of TCCSUP, no bands, and THP-1, which presented an additional smaller band. The predicted MM of native SSC5D is ~165 kDa, still we were expecting a band with a superior size due to the predicted heavy glycosylation. This unexpected result is consistent with alternative splice events that are common feature in SRCR members (Sarrias et al., 2004). Additionally, we also evaluated SSC5D expression in the primary immune cells PBL, monocytes, macrophages and dendritic cells (Fig. 3.2).

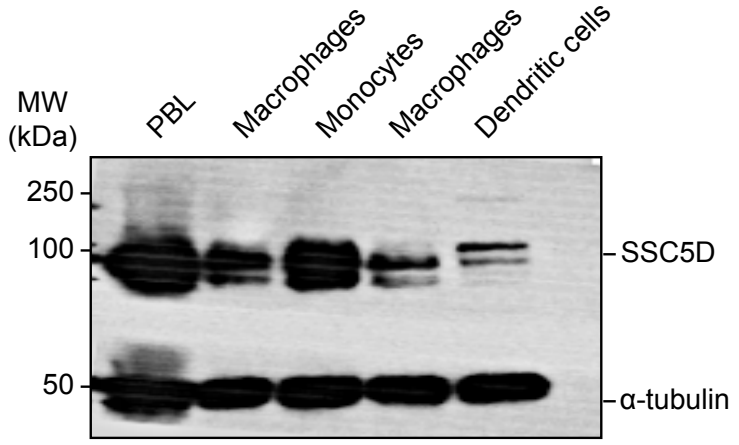

Figure 3.2. SDS-PAGE (7.5%) immunoblotting of the primary cells using the anti-SSC5D antibody followed by peroxidase-conjugated goat anti-rabbit IgG antibody.

Concerning primary cells, we observed two bands representing SSC5D expression with a similar pattern to THP-1. The only exception were dendritic cells where we could observe one band above 100 kDa and an additional faint band at approximately 250 kDa, which could correspond to native full-length SSC5D. Altogether, we conclude that

SSC5D is widely expressed and that a smaller alternative isoform is preferentially expressed over the full-length protein in the cells and conditions analysed.

IHC analysis revealed that SSC5D is preferentially expressed in epithelial cells

To better understand the function of SSC5D, we performed a IHC assay to get detailed information about the localization of this receptor in several human tissue sections (Fig. 3.3).

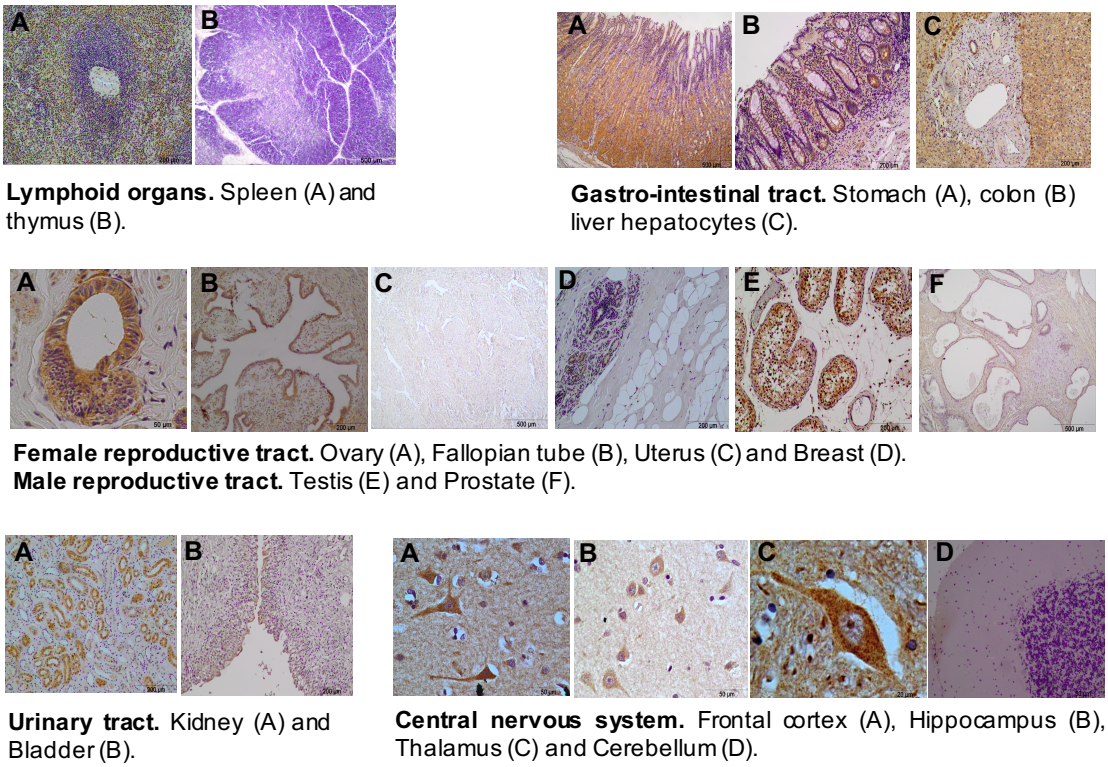

Figure 3.3. Immunohistochemical localization of SSC5D in human tissues. The tissues were stained using a polyclonal antibody directed against SSC5D and counterstained with Mayer's hematoxylin. A positive signal is shown by brown colour.

In the gastrointestinal tract (**colon** and **stomach**), SSC5D is expressed in goblet cells, which are glandular epithelial cells that secrete mucins and other mucus components to form a mucosal barrier. This barrier is important to prevent the bacteria invasion of epithelial surface and trigger inflammatory responses (Johansson et al., 2013). In the **liver**, SSC5D is expressed in hepatocytes, the metabolic cells that are involved in diverse biochemical functions and in the synthesis and secretion of blood proteins, cholesterol, and bile components, among others (Hoekstra et al., 2013). The

expression of SSC5D in the female reproductive tract, is confined to the **fallopian tube** and **ovary follicular** epithelia. Intriguingly, there is no evidence of SSC5D expression in **uterus**. SSC5D is also expressed in the **breast** lobules (milk producing glands). In **testis**, SSC5D is expressed in the spermatogencic cells of the seminiferous tubules (epithelial cells). We also observed the expression of SSC5D in the urinary system, namely in the **kidney** distal collecting ducts (epithelial cells), which is in agreement with its mouse homologue reported results (Miró-Julià et al., 2014). However, in the **bladder** the expression was not so obvious. In the **central nervous system**, the frontal cortex, hippocampus and thalamus exhibit an intense staining in neurons, but no staining in glial cells. In the cerebellum no expression of SSC5D was observed. In lymphoid organs, SSC5D is expressed in a diffuse way in the red pulp of **spleen**, a region containing mainly composed of innate immune cells. Conversely, in **thymus** there is no SSC5D expression.

SSC5D is highly expressed in syncytiotrophoblast

SSC5D is highly expressed in chorionic villi (foetal part), mainly in the cytoplasm of syncytiotrophoblast cells (the outer layer of trophoblast). Interestingly, in a placenta with an undetermined infection, SSC5D expression is undetectable (Fig. 3.4).

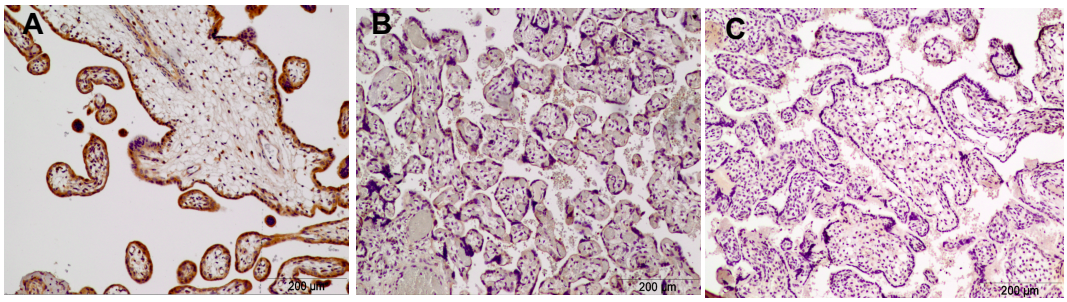

Figure 3.4. Immunohistochemical localization of SSC5D in a typical placenta **(A)** and in a placenta with an unspecified infection **(B)**. Negative control rabbit pre-immune serum **(C)**. Tissue sections were stained using a polyclonal antibody against SSC5D and counterstained with Mayer's hematoxylin. A positive signal is shown by brown colour.

SSC5D is intracellularly expressed in E6.1 cells

The protein sequence of SSC5D predicts a signal peptide (http://www.cbs.dtu.dk/services/SignalP/) that usually directs proteins to the secretion apparatus. In the secretory pathway, proteins can be secreted, target to certain

organelles (endoplasmic reticulum, Golgi or endosomes) or be inserted into the membrane. In addition, SSC5D can be attached to the cell membrane by binding integral membranes. Therefore, to assess whether the protein could be on the cell surface, we performed a flow cytometric analysis using a cell line (E6.1) positive for SSC5D expression. The protocol consisted on using two different buffers, one for surface and other for intracellular staining, which contains saponin a detergent used to permeabilize cell membranes. Cell staining was performed with anti-SSC5D and a secondary antibody coupled to phycoerythrin (PE) fluorescent dye. In the permeabilized cells there is a shift on the intensity of PE meaning that SSC5D is exclusively found intracellularly in E6.1 cells and not at the cell membrane (Fig. 3.5).

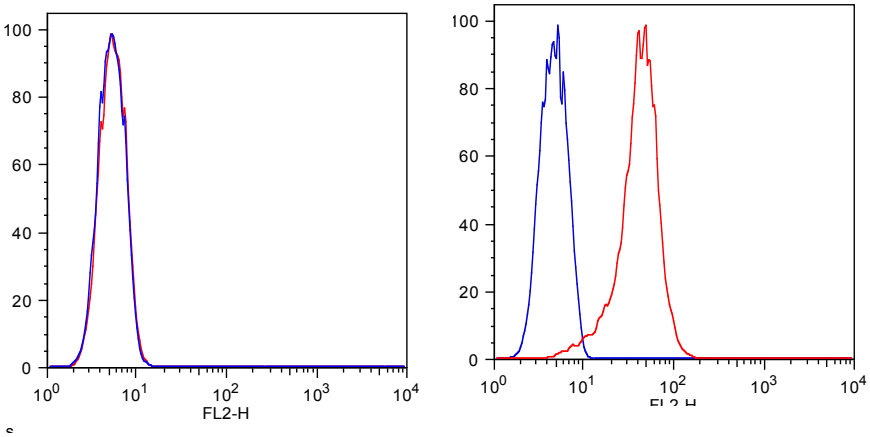

Figure 3.5. Flow cytometric analysis of SSC5D expression in E6.1 cells under nonpermeabilizing (left) or permeabilizing conditions (right). Cells were stained using a polyclonal antibody against SSC5D, followed by the secondary antibody coupled to (PE) to visualize SSC5D (red) or with the (isotype control (IgG), surface) and the PE labelled goat anti-rabbit as a control (blue).

SSC5D localizes to cytoplasmic and nuclear puncta in Caco-2 cell

To study the distribution of SSC5D inside cells, we carried out confocal microscopy analysis of Caco-2 cells, which have been extensively used as a model of the intestinal barrier (Sambuy et al., 2005). We found that SSC5D localises to discrete cytoplasmic puncta (or spots) and not diffuse throughout the cytoplasm. Still, it is unclear whether SSC5D is inside/or associated with endocytic vesicles or whether rather the punctuated distribution represents protein aggregates. Intriguingly, strong nuclear puncta were also observed, in this case likely representing protein aggregates (Fig. 3. 6).

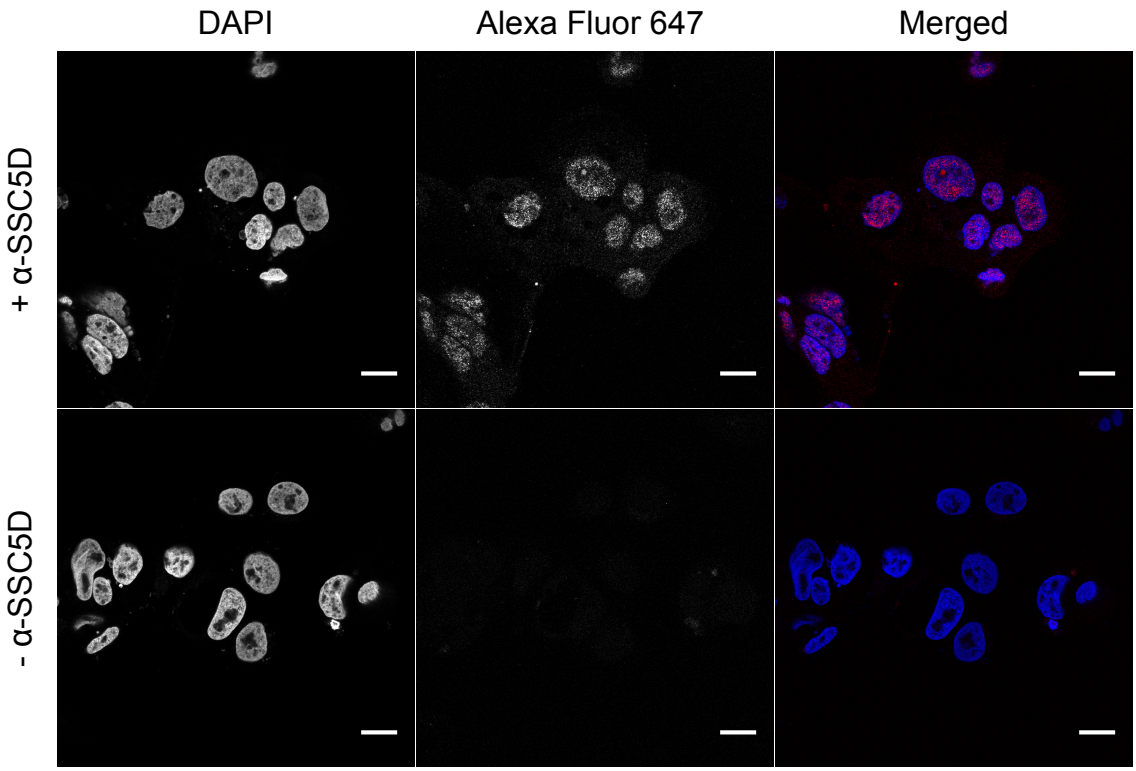

Figure 3.6. Caco-2 cells were imaged using a confocal laser scanning microscope. Cells were stained with anti-SSC5D. Nuclei were counterstained with DAPI (blue) (bars -25 microns).

Generation of SSC5D Knockout Mouse

This task was performed in collaboration with Moises Mallo from the Instituto Gulbenkian de Ciência (IGC), Lisbon. Our strategy to generate a conditional *Ssc5d* KO mouse was through gene targeting using the *Cre/loxP* and *Flp/Frt* recombinase system to selectively inactivate *Ssc5d*. The first step includes the production of a targeting vector to be electroporated into murine embryonic stem (ES) cells and the identification of the targeted ES cell line(s). After establishing the homologous recombinant ES clones, these are microinjected into mice blastocysts to generate chimeric mice. The chimeras can be crossed to an engineered mouse that expresses the bacteriophage gene Cre-recombinase (Cre), which can excise *loxP*-flanked DNA segments (Nagy, 2000) to produce heterozygous mice without the *Ssc5d* allele. Cre recombinase catalyses the recombination between two recognition *loxP* sites, which are 34 bp consensus sequences consisting of a core spacer of 8 bp and two 13 bp palindromic flanking sequences (Nagy, 2000). The heterozygous mice can subsequently be crossed to produce homozygous *Ssc5d* knockout mice.

To construct the targeting vector, we started by retrieving the genomic sequence of the *Mus musculus* homologue of *SSC5D* from the Ensemble database (http://www.ensembl.org/) and inserted the 5'- and 3' homology arm DNA fragments into the PGKneoF2L2DTA vector. The plasmids containing the two fragments of *Ssc5d* were identified by digestions assays, sequenced and sent to the Moises Mallo laboratory to be electroporated into CJ7 embryonic stem (ES) cells.

The targeting vector was composed of:

1) A 5' homology arm of 3.7 kb (exons 1-5) flanked by two *loxP* sites, one at 600 bp upstream the start codon and other after exon 5 to be targeted by the Cre recombinase;

2) A neomycin (neo) resistance cassette to allow the selection of recombinant ES clones. The cassette is flanked by FRT sites to allow its removal by Flp recombination after neo selection;

3) A 3' homology arm of 4 kb (exons 6-8) for homologous recombination.

To establish homologous recombinant ES cell clones, the CJ7 embryonic stem (ES) cells were electroporated with the linearized targeting vector and selected with neomycin. Ninety-six neomycin resistant colonies were picked into 96-well plates and expanded. Next, the genomic DNA of each clone was digested with *Sph*I, *Hind*III/*Spe*I and *Hind*III restriction enzymes in independent reactions to identify targeted clones by Southern blot analysis using a radioactive 5' probe. The radioactive probe hybridizes specifically with the last exon of *Nat14*, the gene preceding *Ssc5d*. Nonetheless, none of the clones exhibited the desired pattern (Fig. 3.7).

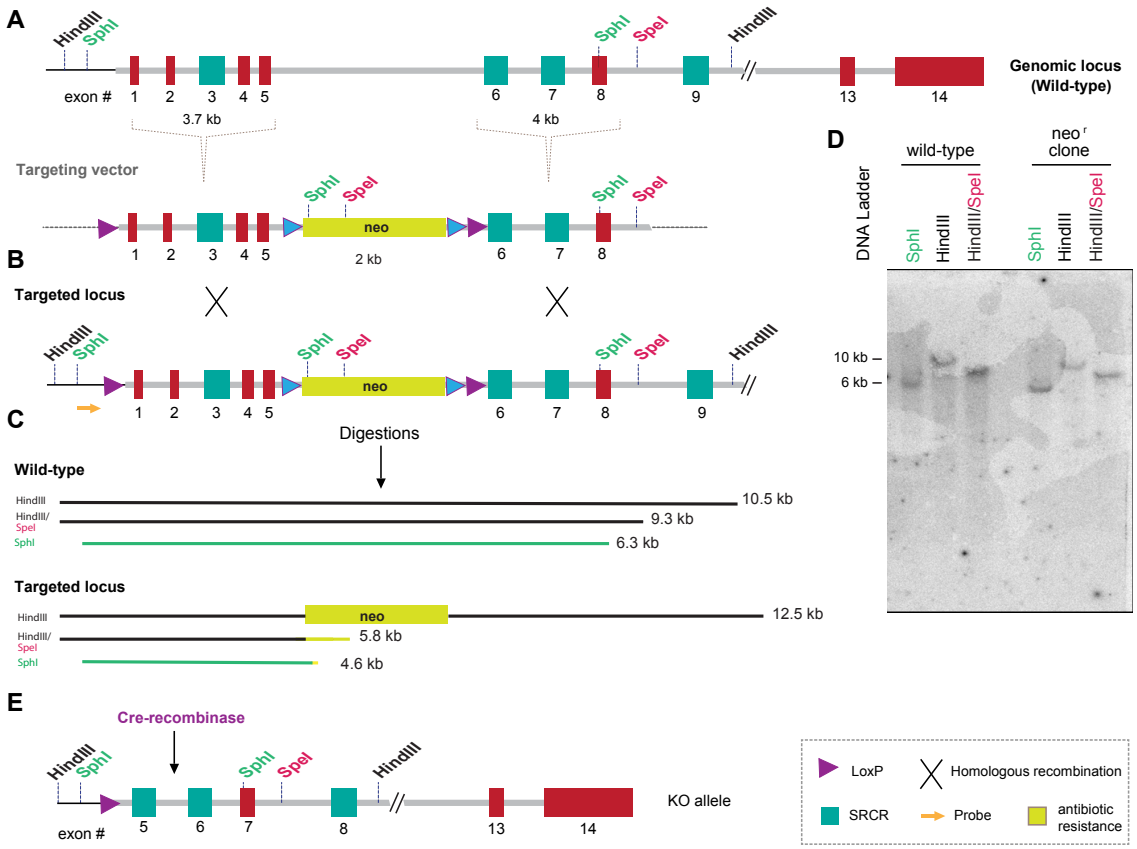

Figure 3.7. Construction of the targeting vector to delete *Ssc5d*. **A.** Fragments of *Ssc5d* genomic DNA (exons 1-5) and (exons 6-8) were amplified by PCR and inserted into the PGKneoF2L2DTA vector to allow the homologous recombination. **B.** Linearized targeting vector. **C.** Expected sizes of the DNA fragments after digestion of the wild-type allele of *Ssc5d* and a homologous recombinant ES clone with *Sph*I, *Hind*III, and double digestion with *Hind*III and *Spe*I restriction enzymes. **D**. Southern blotting analysis of a neomycin resistant clone (right) and a wild type control (left) using a radioactive probe. The band patterns resulting from the digestions are similar in the neomycin resistant-clone and the wild-type allele of *Ssc5d* **E.** Schematic representation of a successful targeted ES clone and the generation of a KO allele through the action of Cre-recombinase.

Given the complexity of the experimental procedures, the ever growing obstacles we were facing and, above all, the surge of a novel and promising new technology, delivering much faster results (CRISPR/Cas9), that was arising and successfully demonstrated in many laboratories, including the lab of Moises Mallo, we decided at this time to terminate this line of work and swapped, already with success, to the CRISPR/Cas9 strategy, performed by other colleagues at our lab. Therefore, these developments are not described in this thesis.

3.4. Discussion

Initial studies carried out in our laboratory revealed the existence of an abundant mRNA transcript corresponding to what is considered the full-length SSC5D, and the existence of other smaller, but less abundant transcripts in placenta, spleen and colon (Gonçalves et al., 2009). However, despite the mRNA levels being indicative of the presence of proteins, mRNA transcript and protein abundance are only partially correlated (Vogel and Marcotte, 2012). This indicates that post-transcriptional regulatory mechanisms, translational control, and protein degradation can also affect protein concentration (Vogel and Marcotte, 2012). Therefore, to analyse the native expression of the SSC5D protein we used a novel antibody against SSC5D to determine 1) the expression of SSC5D in cells from diverse origins using immunoblotting and 2) the distribution of SSC5D in human tissues using immunohistochemistry.

Immunoblotting revealed the existence of a 100-kDa protein from cells of immune origin (T- and B- cells, monocytic and myeloid) and from many tested cells of distinct organ origin. However, the molecular weight corresponding to SSC5D was not the expected for its full-length expression (165 kDa), which indicates that in these cells we may be observing an alternative spliced isoform of SSC5D. In addition, in THP-1 and primary cells (PBL, monocytes and macrophages) an additional smaller band is detected, suggesting the existence of additional alternative spliced isoforms. Interestingly, the expression of SSC5D in dendritic cells follows a different pattern, where we can observe three bands including a faint band around 200 kDa, which suggests the expression of the heavily glycosylated full-length SSC5D.

Alternative spliced variants are common in several members of the SRCR-SF and are often linked to distinct biological events. For instance, one particular isoform of CD163 is insensitive to glucocorticoid stimulation in clear contrast with other CD163 variants (Hoegger et al., 1998). Additionally, the alternative variant of CD6 that lacks the third SRCR does not localize to the immunological synapse (Castro et al., 2007).

Our immunohistochemistry analysis revealed that SSC5D is mainly expressed in epithelial and secretory cells from reproductive and mucosal tissues. This expression pattern much resembles DMBT1 (Holmskov et al., 1999), which has an important role in tissue homeostasis and in pathogen clearance in mucosal tissues (see general introduction). The expression of mouse *Ssc5d* in kidney was already demonstrated in a recent study, where its expression was shown to be up-regulated in a model of urinary

tract infection (Miró-Julià et al., 2014). Additionally, SSC5D is also found in the brain like CD6 (expressed in basal ganglia and cortex cerebellum) (Mayer et al., 1990) and DMBT1, whose down-regulation was related to a higher probability of brain tumours (Mollenhauer et al., 1997).

The spleen is the principal organ responsible for the detection of blood-borne bacterial, viral and fungal pathogens (Bronte and Pittet, 2013). It is divided into two regions called the red pulp, where SSC5D is expressed, and white pulp, which are separated by an interface named the marginal zone (MZ) (Macneal, 1929; Bronte and Pittet, 2013). The white pulp contains T and B cells, which generate antigen-specific immune responses against blood pathogens. Conversely, the red pulp is composed of innate immune cells such as resident macrophages that phagocytose aging red blood cells and regulate iron recycling and release, natural killer T cells that sense lipid antigens, and a reserve of monocytes and dendritic cells (Bronte and Pittet, 2013). In addition, the red pulp has a unique subset of B cells called innate response activator (IRA), which are the main producers of GM-CSF (Bronte and Pittet, 2013). The unique expression of SSC5D in the region containing innate immune cells hints towards its possible role as an innate immune receptor against blood-derived pathogens.

The female reproductive tract consists of several histologically distinct sites and can be divided into a lower tract (vagina and ectocervix) and an upper tract (endocervix, uterus Fallopian tubes and ovaries). The expression of SSC5D was observed in fallopian tubes and ovary epithelia, but was absent in the uterus. However, in HeLa cells (Cervix Adenocarcinoma Cells) the expression of SSC5D was observed, probably indicating that SSC5D is also expressed in the cervix (no tissue section was available). The female reproductive mucosa varies in the lower and upper tract. Accordingly, in upper tract mucosa it is composed of single-layered columnar epithelium, whereas the lower tract is covered by a stratified squamous epithelium, which potentially provides a more effective barrier against invading pathogens than the columnar epithelium (Trifonova et al., 2014).

Epithelial cells, stromal fibroblasts and leukocytes are the main immune components of the female reproductive tract; however, the distribution of leucocytes is also different in the lower and upper tracts (Givan et al., 1997). For instance, T cells, the most abundant cell leukocyte in the reproductive tract, are richer in the lower tract, whereas granulocytes and NK cells are more abundant in the upper tract (Wira et al., 2015). In addition, PRR such as TLR2, TLR4, cytoplasmic RIG as well as NLR are also unevenly distributed. These PRR are more abundant in the upper tract than in the lower tract,

suggesting a mechanism to minimize responses against commensal bacteria and an effective response to pathogenic bacteria in the upper tract, more sensitive to bacteria (Pioli et al., 2004; Ghosh et al., 2013; Wira et al., 2015). The expression profile of cytokines is also different, with more pro-inflammatory cytokines, *e.g.*, IL-1β and IL-6, being expressed in the upper tract, where the environment should be sterile (Wira et al., 2015). The secretion of mucins by the endocervical epithelial cells is also an important immune mechanism to protect epithelia from the contact with pathogens such as HIV (Wira et al., 2015). It is therefore tempting to speculate that SSC5D may have a role as a PRR, namely during viral invasion. The breast expression of SSC5D is also of interest as lactating mothers are more prone to bacterial infections.

Currently, the **placenta** is viewed as an immune regulatory organ that modifies maternal immunologic responses to microorganisms (Racicot et al., 2014). Accordingly, foetal-placenta immune responses must be tightly controlled to prevent pregnancy complications that often result from exacerbated inflammatory reactions. The expression of SSC5D in syncytiotrophoblast epithelial cells, which are in direct contact with maternal blood, may suggest that SSC5D senses microbes that escape cervix protection and reach the uterus, or that are present in maternal blood (Racicot et al., 2014). In addition, SSC5D may also function as damage receptor and promote tissue repair through the interaction with host molecules. Accordingly, a recent study showed that DMBT1 is abundantly observed in injured syncytiotrophoblast areas and was suggested to contribute to tissue repair (Reichhardt et al., 2016). Curiously, SSC5D is apparently absent in a placenta having an infection of unknown aetiology. One possible explanation is that SSC5D is downregulated when challenged with specific microbes. For instance, the treatment of whole blood with *E. coli* results in a significant down-regulation of TLR1 and TLR6 in monocytes and granulocytes (Zarember and Godowski, 2002). Similarly, TLR5 and TLR6 are also down-regulated by Poly(I:C), an analogue of viral dsRNA, and a ligand of TLR3 in primary lung epithelial cells (Ritter et al., 2005). One other study observed that the stimulation of TLR2 with *Borrelia burgdorferi* (responsible for Lyme disease) mediated the down-regulation of TLR5 (recognizes flagellin) (Cabral et al., 2006) in monocytes. The authors suggested that the TLR expression patterns may change in response to diverse environments, including inflammatory conditions, and that these changes might be useful for either the pathogen or the host (Cabral et al., 2006).

The bioinformatics analysis of SSC5D protein sequence does not predict a transmembrane region (Gonçalves et al., 2009). Still, SSC5D can be attached to the

membrane lipid layer through noncovalent interactions with membrane proteins. To test this hypothesis, we used a cell line positive for SSC5D expression (E6.1) and observed SSC5D expression staining under non-permeabilization conditions, and after cell membrane permeabilization. However, we only observed SSC5D intracellularly. This suggests that SSC5D is not expressed at the cell surface and that potential interactions with cell membrane proteins will be of transient nature or it would require an intermediate.

Using confocal microscopy, we noticed a punctuated distribution of SSC5D in the cytoplasm (probably within vesicles) and in the nucleus of Caco-2, colon-derived epithelial cells. The cytoplasmic distribution of SSC5D in low-density Caco-2 cells is similar to that of hensin (rabbit homologue of DMBT1) in intestinal crypt cells (intestinal glands) (Vijayakumar et al., 1999). The intestinal epithelium is held together by tight junctions creating a barrier that difficult the entry of antigenic agents and is divided into two compartments the crypts, where stem cells proliferate and differentiate; and the villi, mainly composed of mature adsorptive cells (Van der Flier and Clevers, 2009). In one study, hensin was found inside vesicles in the crypts, and associated to the extracellular matrix in the villi (Vijayakumar et al., 1999). This study suggested that hensin was associated to the terminal differentiation of epithelia (Vijayakumar et al., 1999). As SSC5D is highly expressed in the intestine and has cytoplasmic distribution similar to DMBT-1, it would be of interest to evaluate SSC5D role in epithelial differentiation.

Images of confocal microscopy also showed SSC5D in the nucleus; however, the protein sequence of SSC5D does not carry a nuclear localization signal to direct it from the cytoplasm to the nucleus (Garcia-Bustos et al., 1991), so this observation was unexpected and we have not yet found a convincingly explanation for this effect.

Adding to this first description of the expression of SSC5D in different organs and tissues, and because SSC5D seems to be expressed in many different body locations by diverse immune and non-immune cells, it was important to develop a mouse model deficient of the expression of *Ssc5d*. However, the strategy we first considered was not successful, as we could not obtain recombinant ES clones to generate chimeras. Nevertheless, using the new gene-editing technology CRISPR-Cas9 (Doudna and Charpentier, 2014), two independent mouse lines of *Ssc5d* KO have been very recently developed by our laboratory.

Despite no functional results being as yet available, we can observe that *Ssc5d* KO mice are viable. In light of what is now known about DMBT1, regarding functions in pathogenic recognition and mucosal homeostasis, it will be noteworthy to explore the function of SSC5D during pathogenic challenges and the susceptibility of *Ssc5d*-defficient mice to different stimuli.

3.5. References

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General discussion

Discussion

Functional and structural studies have shown that proteins of the SRCR superfamily are important in the host defence machinery. For example, they are implicated in the differentiation and activation of lymphocytes, in the protection against broad microbial agents, in the modulation of inflammation and in tissue repair mechanisms. Despite the common presence of the highly conserved SRCR domain, this large family constitutes a particularly heterogeneous group that includes transmembrane and soluble glycoproteins that are expressed in different cell types and body locations. Moreover, some functions of the SRCR proteins are not attributed to their SRCR domains but to other structures or regions within the protein (Martínez et al., 2011). On the other hand, very similar proteins, for example CD163L1 is not capable of bacteria recognition, while the highly similar protein CD163 binds bacteria through the second SRCR domain (Grønlund et al., 2000; Fabriek et al., 2009; Moeller et al., 2012). These findings suggest that the distinctive presence of the highly conserved SRCR domain might not be a requisite for the function of a SRCR member, and that we cannot predict the function of a singular SRCR member based only on its common structural features.

Notwithstanding these arguments, there is some evidence to suggest that the SRCR domains are responsible for protein-protein interactions and that are correlated to protein function. For instance, the T cell transmembrane glycoprotein CD6 binds the surface cell ligand CD166 through its third SRCR member and this interaction is important to localize CD6 in the immunological synapse (Hassan et al., 2004; Castro et al., 2007). The structural interaction of these proteins was resolved recently and revealed that the SRCR domains 1 and 2 also indirectly favour this interaction (Chappell et al., 2015). The CD6-CD166 interaction induces a series of signal transduction cascades that endow CD6 with a role in T cell modulation during APC-TCR contacts. Still, as it happens with other SRCR-related interactions, the extent and biological relevance of such interaction remains to be fully apprehended.

The cloning and first analyses of SSC5D in our laboratory revealed the existence of a new soluble glycoprotein member of the SRCR-SF composed of 5 SRCR domains and a mucin-like domain (Gonçalves et al., 2009). Given that we are now working with a novel protein having no information whatsoever about its function, we proposed at the start of this study to cover the major steps in its characterization, namely its tissue distribution and its functional binding to other cells, self and/or non-self. Additionally, a

central aim was to develop a knockout mouse to infer the functional role of Ssc5d in models of infection and/or inflammation.

For characterizing any functional effects, we were required to produce and purify the SSC5D protein. In chapter 1, we thus focused on the production of the moieties corresponding to the SRCR containing domains (N-SSC5D) and the mucin-like domain (C-SSC5D) to independently analyse their binding capacity to several cells surfaces. The rationale was to understand whether the SRCR domains were, like CD6, capable to bind cells surfaces and provide some evidence about the existence of a cognate ligand. However, some substantial differences exist between CD6 and SSC5D, namely the fact that CD6 is a transmembrane protein shown to be directly involved in cell-cell contact and in the activation of signalling pathways as discussed earlier, whereas SSC5D is a soluble/secreted protein expressed by a variety of cells, including epithelial and immune cells (chapter 3). We thus additionally produced the soluble recombinant Spα because it represented a more suitable comparable protein to SSC5D to analyse putative interactions.

The proteins were assembled in a tetrameric form to increase the affinity for cell surface receptors (a previously strategy developed by our laboratory). Unfortunately, we did not detect significant N-SSC5D binding to any of the cells analysed. However, at this stage it is premature to discard a potential interaction of N-SSC5D with cell surface receptors as a wider analyse could deliver potential interactions. In addition, we did not explore other experimental conditions that could increase the sensitivity of this method. Regarding the mucin-like domain (C-SSC5D), the production of large quantities of soluble N-SSC5D was hampered by technical difficulties, namely the formation of massive aggregates during the purification steps, which we relate to the complexity of the *O*-glycosylation events of recombinant glycoproteins (Dwek R., 1996). Often the reduced availability of proteins (recombinant or native) poses an obstacle to complete experimental protocols. Hence, the development of more sensitive techniques are also hindered by this limitation.

Not having obtained evidence for interactions of SSC5D with receptors in endogenous cells, we hypothesized that it might have a role in the identification of foreign cells, namely bacteria, as had been demonstrated for other SRCR proteins. In chapter 2, we thus explored this possibility and developed an SPR-based assay for the rapid and sensitive detection of bacterial binding to the SRCR domains of SSC5D. It must be noted that studies concerning the mouse homologue of SSC5D were published at the time of this work, so these and other reports, including those focusing on Spα and its

ability to bind bacteria, constitute an additional and more solid background from where to develop better suited experimental methods.

The application of label-free detection mechanisms based on optical sensors has been gathering much attention and is now viewed as an alternative to conventional protocols used to study biomolecular interactions. Accordingly, SPR biosensors allow rapid, direct and real-time observation of the protein-bacteria interaction and usually require less protein and bacteria to perform multiple analyses. Moreover, since bacteria are not static and are usually carried by flowing fluids (water, urine, or blood), SPR-based analyses better represent the dynamics of pathogenic interaction, when compared with traditional methods (Zagorodko et al., 2015). Therefore, in this work we asked whether this tool was suitable to study the interaction of surface-immobilized N-SSC5D with different types of bacteria.

As SSC5D is highly expressed in placenta, we selected types of bacteria, namely the Gram-negative *E. coli* O18:K1:H7 strains RS218 and IHE3034 as well as the Gram-positive *L. monocytogenes,* which are able to cross the placental barrier and cause pregnancy and/or neonatal complications. The *E. coli* K1 strains are the main responsible for neonatal gram-negative bacillary meningitis and have several virulence factors including the outer membrane protein A and invasins (IbeA and IbeB) that promote the invasion of the blood-brain barrier (Yao et al., 2006). Nonetheless, the RS218 strain has additional virulence factors such as P fimbria and the Hek outer membrane protein that mediate adherence to host cells (Yao et al., 2006). In the SPR assay we observed that N-SSC5D could bind both strains of bacteria, but it bound slightly better RS218, which hinted to a possible discrimination of bacteria surface molecules. Interestingly, N-SSC5D could not bind heat-killed IHE3034, which suggests that intact surface structures are needed for the interaction to occur. The binding affinity of N-SSC5D to *L. monocytogenes* was revealed by the SPR assay and was inferior to the binding to the *E. coli* strains, suggesting that N-SSC5D can differentiate between types of bacteria.

As described in several studies, some SRCR-SF members bind to purified LPS, LTA and/or PGN; however, it is unclear whether these molecules are the ligands on the surface of intact bacteria (Areschoug and Gordon, 2009). It is possible that other microbial proteins are ligands for these PRR. For instance, the binding of the scavenger receptor-A (SR-A) to *Neisseria meningitidis* is independent of surface LPS, as shown by using a lipid A-deficient isogenic mutant of *N. meningitidis* (Peiser et al., 2002). Later studies identified *N. meningitidis* outer membrane proteins as ligands for

SR-A (Areschoug and Gordon, 2009). In addition, as it happens with TLR, SRCR can have a wide range of ligands (Brubaker et al., 2015). This study emphasizes that differences in the ligands recognized by the SRCR receptors might exist. Therefore, sensitive studies using SPR multi-channel devices to detect the binding of SRCR receptors to whole bacteria may help in the search of the bacterial structures responsible for specific SRCR-bacteria interactions.

The function of a protein depends on its location, so we devoted chapter 3 for a wide characterization of SSC5D expression. We observed that SSC5D is mainly expressed by the epithelial cells in several organs, especially at mucosal sites. Epithelial cells at the mucosal surfaces form a physical barrier that is protected by several immune effector systems that include the secretion of mucin glycoproteins, for example by intestinal goblet cells, that coat the intestinal epithelia to minimize bacterial-epithelial contact, and the production of antibacterial components such as α-defensins, collectins and soluble (s) IgA (McGuckin et al., 2011; Hooper et al., 2012). In addition, epithelial cells can directly respond to pathogenic and opportunistic agents via PRR, such as TLR and NLR, and trigger immune responses (Bulek et al., 2010; McGuckin et al., 2011). DMBT1 at mucosal surfaces was shown to interact with pathogens, *e.g.*, *H. pylori*, *S. mutans* and Influenza A virus, as well as antimicrobial components, including sIgA, MUC5 and lung surfactant proteins SP-D and SP-A (Kang and Reid, 2003). This capacity suggests that DMBT1 can directly bind pathogens or work in synergy with other host proteins to effectively clear microorganisms. In addition, DMBT1 also binds the host galectin-3 (Rossez et al., 2011), a glycoprotein involved in the regulation of cell proliferation, differentiation and apoptosis (Dumic et al., 2006).

The expression profile of SSC5D (chapter 3), the ability to bind bacteria through the N-SSC5D domain (chapter 2) which contains the scavenger domains, and the capacity of its mouse homologue to bind the endogenous extracellular proteins laminin, galectin-1 and -3 (Miró-Julià et al., 2011; Miró-Julià et al., 2014) much resembles some of the DMBT1 features. However, unlike DMBT1 and a few other SRCR molecules, there were no knockout animals developed previously to allow us a better comparison with the function of the other SRCR proteins. We are about to fill that gap as we have just recently developed two independent lines of *Ssc5d* mice, and hopefully we will assemble important data regarding the function of Ssc5d in physiological conditions and in models of disease. The results obtained in this thesis predict a role for SSC5D in mucosal immunity and possibly in epithelial cell homeostasis. Therefore, future experiments, especially using our recently generated Ssc5d KO mice may open new avenues for studying the functional relevance of this intriguing immune receptor.

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